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Two *ex situ* fungal technologies to treat contaminated soil

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ACADEMIC DISSERTATION

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"She is a wild and resourceful beast given to fits a rage. And now that we are provoking her beyond endurance, she is starting to seek her revenge"

John Grant, Co-Opportunity

To my brother *Rober* for his endless support

Contents

List of original publications

This thesis is based on the following publications, which are referred in the text by Roman numerals I-V.

I **Valentín L.,** Feijoo G., Moreira M.T. and Lema J.M. 2006. Biodegradation of polycyclic aromatic hydrocarbons in forest and salt marsh soils by white-rot fungi. International Biodeteroration and Biodegradation. 58: 15-21.

II **Valentín L.,** Lú-Chau T.A., López C., Feijoo G., Moreira M.T. and Lema J.M. 2007. Biodegradation of dibenzothiophene, fluoranthene, pyrene and chrysene in a soil slurry reactor by the white-rot fungus *Bjerkandera* sp. BOS55. Process Biochemistry. 42: 641-648.

III **Valentín L.,** Kluczek-Turpeinen B., Oivanen P., Hatakka A., Steffen K. and Tuomela M. 2009. Evaluation of basidiomycetous fungi for pretreatment of contaminated soil. Journal of Chemical Technology and Biotechnology. 84: 851-858.

IV Winquist E., **Valentín L.,** Moilanen U., Leisola M., Hatakka A., Tuomela M. and Steffen K.T. 2009. Development of a fungal pre-treatment process for reduction of organic matter in contaminated soil. Journal of Chemical Technology and Biotechnology. 84: 845-850.

V **Valentín L.,** Kluczek-Turpeinen B., Willför S., Hemming J., Hatakka A., Steffen K. and Tuomela M. 2010. Scots pine (*Pinus sylvestris*) bark composition and degradation by fungi: Potential substrate for bioremediation. Bioresource Technology. 101: 2203-2209.

Author's contribution

I The author designed and executed the experimental work, analyzed and interpreted the results, made the statistical analyses, and wrote the article together with the other authors.

II The author designed and executed the experimental work, except for some soil slurry fermentations, analyzed and interpreted the results, and collaborated with the other authors to write the article.

III The author was responsible for the experimental work, planned and executed all of the experiments, except for the screening with LOM soil, analyzed and interpreted the results, wrote the article and is the corresponding author.

IV The author participated in the design and execution of the experimental work in the large-scale reactor, made the enzyme analyses and followed the respiratory activity of the soil experiments in the large-scale reactor. She collaborated in the analyses and the interpretation of the results and in the writing of the article.

V The author was responsible for the experimental work and is the corresponding author. She also planned and executed all of the experiments, except for the chemical analyses of lignin and polysaccharides, and the GC-MS analyses of the extractives. She analyzed and interpreted the results and wrote the article.

Abbreviations

ABTS	2,2´-azinobis(3-ethylthiazoline-6-sulfonate)
ANCOVA	analyses of covariance
ANOVA	analyses of variance
ASE	accelerated solvent extraction
BaP	benzo[a]pyrene
BRF	brown-rot fungi
CEC	cation exchange capacity
CMC	carboxymethylcellulose sodium salt
CPs	chlorophenols
GC - MS	gas chromatography - mass spectrometry
HA	humic acids
HOM	high organic matter
HPLC	high performance liquid chromatography
HS	humic substances
IP	ionization potential
Lacc	laccase
LDF	litter-decomposing fungi
LiP	lignin peroxidase
logK_{ow}	logarithm octanol-water partition coefficient
LOM	low organic matter
LMEs	lignin-modifying enzymes
MiP	manganese independent peroxidase
MnP	manganese peroxidase
OC	organic carbon
OM	organic matter
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCDD/Fs	dibenzo-*p*-dioxins and -furans
PCP	pentachlorophenol
PCPPs	polychlorinated phenoxyphenols
PPCPs	pharmaceutical and personal care products
RBBR	remazol brilliant blue R
SOM	soil organic matter
TNT	trinitrotoluene
UFA	unsaturated fatty acids
VP	versatile peroxidase
WRF	white-rot fungi

Abstract

Wood-degrading basidiomycetes, specifically white-rot and litter-decomposing fungi, are able to degrade a large range of recalcitrant pollutants which resemble the lignin biopolymer. This ability is mainly attributed to the production of lignin-modifying enzymes, which are extracellular and non-specific. Despite the potential of fungi to degrade contaminants, there is still an understanding gap in terms of the technology. In this thesis, the feasibility of two *ex situ* fungal bioremediation methods was evaluated.

Treatment of polycyclic aromatic hydrocarbons (PAHs)-contaminated marsh soil was studied in a stirred slurry-phase reactor. Due to the salt content in marsh soil, fungi were screened for their halotolerance, and the white-rot fungi *Lentinus tigrinus*, *Irpex lacteus* and *Bjerkandera adusta* were selected for further studies. With a soil concentration of 10% (w/v), all the three fungi degraded 40 - 60% of a PAH mixture (phenanthrene, fluoranthene, pyrene and chrysene) in a 100 ml slurry-phase reactor during 30 days of incubation. The process was scaled-up to a 5 litre reactor and glucose concentration, the inoculum type and biomass were optimized for *B. adusta*. Maximum degradation of dibenzothiophene (93%), fluoranthene (82%), pyrene (81%) and chrysene (83%) was achieved with the free mycelium inoculum of the highest initial biomass (2.2 g/l). A glucose concentration of 20 ± 3 g/l enhanced the PAH degradation. Manganese peroxidase (MnP) was produced by *B. adusta* after a lag phase of seven days. In autoclaved soil, MnP was probably the most important enzyme involved in PAH degradation. In non-sterile soil, endogenous soil microbes together with *B. adusta* also degraded the PAHs extensively, suggesting a synergic action between soil microbes and the fungus.

A fungal solid-phase cultivation method to pretreat contaminated sawmill soil with high organic matter content was developed to enhance the effectiveness of the subsequent soil combustion. The preliminary screening of 146 fungal strains showed that 34 strains extensively colonized non-sterile contaminated soil. These fungi belonged to the group of litter-decomposing fungi (28 out of 52) and to white-rot fungi (13 out of 56). Later, the 18 strains selected were characterized by their production of lignin-modifying and hydrolytic enzymes during cultivation on Scots pine (*Pinus sylvestris*) bark. The main enzymes produced by fungi in the bark were MnP and endo-1,4-β-glucanase. Of the six fungi selected for further tests, *Gymnopilus luteofolius*, *Phanerochaete velutina*, and *Stropharia rugosoannulata* were the most active soil organic matter degraders. It was estimated from these results that a six-month pretreatment of sawmill soil would result in a 3.5 - 9.5% loss of organic matter, depending on the fungus applied. The pretreatment process was scaled-up for a 0.56 m^3 reactor, in which mesh plastic tubes filled with *S. rugosoannulata* growing on pine bark were introduced into the soil. From these tubes, *S. rugosoannulata* formed extensive mycelium that colonized the soil. The fungal pretreatment resulted in a soil mass loss of 30.5 kg, which represents 10% of the original soil mass (308 kg).

The suitability of pine bark as fungal substrate for bioremediation was studied. Thereby importance was attached to the chemical composition of bark. Bark contained more lignin (45%) than cellulose (25%) or hemicellulose (15%), and the most abundant extractives belonged to the group of resin acids (especially dehydroabietic acid), followed by sitosterol. A high content of the unsaturated fatty acids (UFA), namely oleic, linoleic and linolenic acid, was also characteristic of the bark. Both of the studied fungi (*P. velutina* and *S. rugosoannulata*) degraded all of the bark main biopolymers simultaneously. Despite the fact that bark contains several antimicrobial compounds, fungi were able to colonize it extensively and to produce enzymes, especially MnP and endo-1,4-β-glucanase, during the cultivation process. Interestingly, UFA degradation coincided with the peaks of lignin loss and MnP production. This result suggested that MnP-mediated lipid peroxidation may have played a role in lignin degradation.

Fungal technologies to treat contaminated soil provide an alternative to conventional technologies (e.g., stabilization and combustion). *Ex situ* slurry-phase fungal reactors might be applied in cases when the soil has a high water content or when the contaminant bioavailability is low; for example, in wastewater treatment plants to remove pharmaceutical residues from sludges. Fungal solid-phase bioremediation is a promising remediation technology to *ex situ* or *in situ* treat contaminated soil in such cases where bacterial bioremediation is not possible. For fungal bioremediation, Scots pine bark is a suitable substrate for fungal growth and promoter of the production of oxidative enzymes, as well as an excellent and cheap natural carrier of fungal mycelium.

Resumen (Abstract in Spanish)

Los hongos saprófitos degradan el material leñoso y los restos vegetales gracias a la producción de diversas enzimas oxidativas e hidrolíticas. Debido a la naturaleza extracelular y no específica de las enzimas, los hongos son también capaces de atacar contaminantes orgánicos cuya estructura molecular se asemeja a la del biopolimero más complejo de la madera, la lignina. Algunos ejemplos de estos compuestos son los hidrocarburos aromáticos policíclicos (HAPs) o los compuestos clorados (pentaclorobifenilos or pentaclorofenol). A pesar de esta capacidad degradadora, la información disponible en relación a la tecnología fúngica para el tratamiento de suelos contaminados es aún escasa. A fin de aumentar el conocimiento de la biorremediación mediante la aplicación de los hongos, este trabajo se centro en el desarrollo y el escalado de dos tecnologías fúngicas *ex situ*: el reactor en fase suspensión (slurry) y la biopila en estado sólido.

En primer lugar, se estudió el tratamiento fúngico de un suelo de marisma contaminado con HAPs en un sistema en fase suspensión con una carga de suelo del 10%. Inicialmente se investigó la capacidad degradativa de nueve cepas de hongos a escala pequeña (100 ml) con el fin de seleccionar aquellos hongos con mayor adaptabilidad a las condiciones salinas del medio. De este estudio se seleccionaron tres hongos de la podredumbre blanca, *Lentinus tigrinus*, *Irpex lacteus* y *Bjerkandera adusta*, por degradar significativamente (40 - 60%) los HAPs (fenantreno, fluoranteno, pireno y criseno) tras 30 días de incubación y por tolerar la salinidad del medio. A continuación se realizó el escalado en un biorreactor de 5 l operado con *B. adusta* con el objetivo de evaluar el efecto de diversos factores tales como la concentración y el tipo de inóculo de hongo, la concentración inicial de glucosa y la acción de los microorganismos endógenos sobre el potencial degradador de *B. adusta*. Las mayores degradaciones de dibenzotiofeno (93%), fluoranteno (82%), pireno (81%) y criseno (83%) se obtuvieron adicionando 2.2 g/l de micelio homogeneizado del hongo. Asimismo, la aportación inicial de 20 ± 3 g/l de glucosa al reactor ejerció un efecto positivo sobre la degradación de los HAPs. La enzima manganeso peroxidasa (MnP), cuya producción se inició tras siete días de cultivo, se vinculó a la degradación de HAPs. También se observó una acción sinérgica entre los microorganismos endógenos y el hongo en relación a la degradación de HAPs.

En segundo lugar, se desarrolló un pretratamiento en estado sólido con la finalidad de mejorar la eficiencia del proceso de combustión de suelos de aserraderos con elevada carga orgánica. El estudio preliminar de 146 cepas fúngicas dió lugar a la selección de 34 cepas con excepcional capacidad para crecer en suelo contaminado. Posteriormente, se estudió la producción de las enzimas ligninolíticas e hidrolíticas de 18 hongos durante su cultivo en corteza de pino silvestre (*Pinus sylvestris*). Se encontró que en este tipo de material lignocellulósico los hongos exhiben mayor actividad de las enzimas MnP y endo-1,4-β-glucanasa. Estos estudios derivaron en la selección de seis hongos, de los cuales tres, *Gymnopilus luteofolius*, *Phanerochaete velutina* y *Stropharia rugosoannulata*,

consiguieron elevadas tasas de degradación de materia orgánica del suelo (3.5 - 9.5%) tras un periodo de seis meses. A continuación se realizó el escalado del proceso en un reactor de 0,56 m^3 el cual se inoculó con cultivos de *S. rugosoannulata* crecido sobre corteza de pino. Este inóculo se introdujo en unos tubos perforados que fueron insertados horizontalmente en el suelo. Se observó claramente como las hifas del hongo colonizaron el suelo formando un extenso micelio alrededor de los tubos. El pretratamiento del suelo resultó en la pérdida de 30,5 kg de masa de suelo, representando alrededor del 10% de la masa original (308 kg).

Finalmente se realizó un estudio sobre la composición química de la corteza de pino y su viabilidad como material lignocellulósico para la biorremediación fúngica. Los análisis químicos desprendieron los siguientes resultados en cuanto a la composición de la corteza de pino: 45% lignina, 25% celulosa y 15% hemicelulosa. De entre los extractos solubles en acetona los compuestos en mayor proporción fueron las resinas y el sitosterol. Los ácidos grasos insaturados oléico, linoléico y linolénico constituyeron también una fracción importante del total de los extractos. En cuanto a la degradación de la corteza de pino, los hongos *P. velutina* y *S. rugosoannulata* degradaron los principales biopolímeros de la corteza de pino simultáneamente. Lo más significativo de este estudio fue el elevado grado de colonización de micelio sobre la corteza y la elevada actividad de MnP. Se encontró que la degradación de los ácidos grasos insaturados fue coincidente con la producción de MnP y con la pérdida de lignina. Este resultado sugirió que la peroxidación lipídica mediada por la acción de la peroxidasa pudo haber participado en el ataque a la lignina.

Los resultados que se desprenden de esta tésis ofrecen nuevas oportunidades para el tratamiento sostenible de los suelos contaminados basado en los hongos. El biorreactor *ex situ* puede aplicarse en situaciones en las que el suelo tenga un elevado contenido de agua o una baja biodisponibilidad del contaminante. Por ejemplo este tipo de reactores podrían implantarse en plantas de tratamiento de aguas residuales para el tratamiento de los lodos. La biorremediación fúngica en estado sólido podría ser muy apropiada en aquellos trabajos de descontaminación *ex situ* o *in situ* en los que la aplicación con base bacteriana estaría más limitada. Dado que la corteza de pino fue un excelente sustrato para el crecimiento de los hongos y promotor de la producción de enzimas oxidativas, se recomienda este material para la producción de micelio fúngico en trabajos de descontaminación.

Tiivistelmä (Abstract in Finnish)

Puuta lahottavat kantasienet, erityisesti valkolahosienet ja karikkeenlahottajasienet, pystyvät hajottamaan monia erilaisia vaikeasti hajoavia ympäristömyrkkyjä. Luonnossa nämä sienet hajottavat ligniiniä solunulkoisilla entsyymeillä, jotka ovat epäspesifejä ja sen vuoksi kykeneviä hajottamaan myös muita yhdisteitä, kuten maata pilaavia orgaanisia yhdisteitä. Sieniin perustuvaa puhdistustekniikkaa ei kuitenkaan ole vielä kaupallisesti saatavilla. Tässä väitöskirjassa tutkittiin kahden sienitekniikan toteuttamiskelpoisuutta pilaantuneen maan puhdistamiseksi.

Polyaromaattisilla hiilivedyillä (PAH) pilaantuneen marskimaan käsittelyä tutkittiin sekoitettavassa bioreaktorissa. Koska marskimaa sisältää suolaa, sienet seulottiin ensimmäisessä vaiheessa suolan sietokyvyn perusteella, jolloin löydettiin kolme korkeaa suolapitoisuutta sietävää valkolahosientä: *Lentinus tigrinus*, *Irpex lacteus* ja *Bjerkandera adusta*. Nämä sienet hajottivat tehokkaasti PAH-seosta (fenantreeni, fluoranteeni, pyreeni, kryseeni) kolmessakymmenessä päivässä 100 ml kokoisessa reaktorissa, jossa oli 10 % maata ja loput nestettä. Prosessia testattiin myös viiden litran reaktorissa *B. adusta*-sienellä, jolloin glukoosin pitoisuus nesteessä, sienisiirroksen tyyppi ja biomassa optimoitiin. Kun sienisiirrosta lisättiin reaktoriin maksimimäärä (2,2 g/l) saavutettiin myös suurin hajotusteho: dibentsotiofeenista hajosi 93 %, fluoranteenista 82 %, pyreenistä 81 % ja kryseenistä 83 %. Glukoosin optimipitoisuus PAH-yhdisteiden hajotukselle oli 20 ± 3 g/l. *B. adusta* alkoi tuottaa mangaaniperoksidaasia (MnP) reaktorissa seitsemän päivän kasvatuksen jälkeen ja se oli todennäköisesti tärkein PAH-yhdisteitä hajottava entsyymi maassa, jossa kasvoi pelkästään *B. adusta*. Epästeriilissä maassa *B. adusta* oletettavasti hajotti PAH-yhdisteitä yhdessä maan omien mikro-organismien kanssa.

Toinen sienitekniikkasovellus kehitettiin pilaantuneelle maalle, jossa orgaanisen aineen pitoisuus on suuri. Tällaista maata esikäsiteltäisiin sienen avulla ennen maan polttoa. Esikäsittelyn tarkoitus on maan orgaanisen aineen vähentäminen ja siten polttoprosessin tehostaminen. Sopivat sienet seulottiin 146 sienikannan joukosta, joista 34 kasvoi hyvin maahan. Näistä sienistä suurin osa eli 28 oli karikkeenlahottajasieniä. Kun parhaiten kasvaneita 18 sienikantaa kasvatettiin männyn (*Pinus sylvestris*) kaarnalla, ne tuottivat ligninolyyttisiä ja hydrolyyttisia entsyymejä; etenkin MnP:ia ja endo-1,4-β-glukanaasia. Kaikkein aktiivisimmin orgaanista ainetta maassa hajottivat *Gymnopilus luteofolius*, *Phanerochaete velutina* ja *Stropharia rugosoannulata*. Tulosten perusteella arvioitiin, että saha-alueen maan esikäsittelyllä saavutettaisiin 3.5 - 9.5 % orgaanisen aineen hävikki kuudessa kuukaudessa sienilajista riippuen. Maan esikäsittelyä testattiin myös 0.56 m^3 reaktorissa, johon sienisiirros lisättiin verkkomaisissa muoviputkissa. Putket sisälsivät kaarnalla kasvavaa *S. rugosoannulataa*, joka levisi putkien raoista maahan koko reaktorissa. Maan massahävikki esikäsittelyssä oli 30,5 kg, eli n. 10 % maan alkuperäisestä massasta (308 kg).

Männyn kaarnan käyttökelpoisuus sienen ravinteeksi maan käsittelyssä arvioitiin, ja kaarnan kemiallinen koostumus määritettiin. Kaarna sisälsi 45 % ligniiniä, 25 % selluloosaa ja 15 % hemiselluloosaa. Uuteaineista yleisimmät olivat hartsihapot (etenkin dehydroabietiinihappo) ja sitosteroli. Tyydyttymättömien rasvahappojen (UFA) pitoisuus oli korkea, koostuen pääasiassa oleiinihaposta, linolihaposta ja linoleenihaposta. Kaarnassa kasvaessaan sekä *P. velutina* että *S. rugosoannulata* hajottivat kaarnan kaikkia makromolekyylejä yhtäaikaisesti. Vaikka kaarna sisältää useita mikrobien kasvua ehkäiseviä yhdisteitä, sienet kasvoivat siinä hyvin tuottaen kasvaessaan erityisesti MnP:ia ja endo-1,4-β-glukanaasia. Tyydyttämättömien rasvahappojen hajoaminen oli samanaikaista kuin ligniinin hajoaminen ja MnP:n tuoton huippukohta. Tulos viittaisi siihen, että lipidiperoksidaatio eli rasvojen hapettuminen edisti osaltaan ligniinin hajoamista.

Pilaantuneen maan käsittely sienitekniikalla tarjoaa vaihtoehtoisen menetelmän vakiintuneille menetelmille, kuten stabilointi ja poltto. Reaktoria voitaisiin hyödyntää tapauksissa, joissa maan vesipitoisuus on suuri tai kun pilaavan yhdisteen biosaatavuus on alhainen, esimerkiksi lääkejäämien poistoon jätevesistä. Pilaantuneille maamassoille sienitekniikkaa voitaisiin soveltaa sekä ex situ että in situ. Hinnaltaan edullinen männyn kaarna sopii sienten kasvualustaksi ja sienisiirroksen kantaja-aineeksi erinomaisesti edistäen hapettavien entsyymien tuottoa.

1 INTRODUCTION

1.1 Soil as a system

Soil is a complex system in its structure and function due to intricate relations between the biotic community and the medium surrounding it. In soil, *de novo* material is produced constantly and, at the same time, organic matter is decomposed, releasing energy and providing nutrients to plants and other organisms (Paul, 2007). Soil organic matter (SOM) is essential in supporting the chemical and physical properties of the soil, thus maintaining soil quality and function.

Microorganisms are mainly responsible for SOM dynamics, but the role of micro-, meso- and macro-fauna is also crucial for assisting microbes in colonizing and degrading the organic matter by physically and chemically altering the soil structure (Coleman and Wall, 2007). The most diverse group of microorganisms living in soil are bacteria followed by fungi and archaea. Torsvik et al. (1998) estimate that total bacterial diversity in pristine soil corresponds to more than 10^4 bacterial types. Common soil bacteria belong to the phyla of *Proteobacteria*, *Firmicutes, Actinobacteria*, *Bacteroidetes, Planctomycetales, Verrucomicrobiales* and *Acidobacteria* (van Elsas et al., 2007). In the uppermost layer of soil, fungi are more abundant in terms of biomass. For example, in grasslands the fungal biomass can reach 2 - 5 tons per hectare (Finlay, 2007). Currently, the number of fungal species in soil is not available. This is mainly due to the difficulties in isolating and characterizing soil fungi by culturing techniques. Recently, new molecular studies on soil fungal diversity, based on polymerase chain reaction (PCR) followed by next-generation sequencing of the internal transcribed spacers (ITS), have facilitated researchers in identifying species more precisely (Buée et al., 2009). Representatives of the traditional phyla of the Fungi Kingdom are found in soil: i) *Chytridiomycota* is represented by plant pathogens and parasites; ii) *Zygomycota* includes parasitic and saprotrophic fungi; iii) *Glomeromycota* includes arbuscular mycorrhiza-forming fungi; iv) *Ascomycota* is the largest group with approximately 50,000 species and, thus, with different ecological roles in the soil; v) in *Basidiomycota* only, the so-called homobasidiomycetes are found in soils which include wood-decaying and litter-decomposing fungi, soil-borne pathogens of crops and forest trees, as well as the ectomycorrhizal fungi of woody plants (Thorn and Lynch, 2007). Wood-decaying and litter-decomposing Basidiomycetes are discussed in more detail in section 1.4.

Humic substances (HS) constitute the major percentage (up to 80%) of SOM originating from the transformation of plant and animal residues and from microbial activity (Senesi and Loffredo, 2001). The exact composition and chemical structure of HS are not yet known, but lignin-derived structures are the main source of HS formation (Shevchenko and Bailey, 1996; Senesi and Loffredo, 2001). In contaminated soil, in addition to degradation, the humification of contaminants (i.e. incorporation to HS

structures) is an important detoxification process (Bollag, 1992; Senesi and Loffredo, 2001). Besides abiotic processes, such as the hydrophobic adsorption of chlorinated compounds, the actions of extracellular enzymes of soil microorganisms may help with the binding of soil contaminants to HS (Bollag, 1992). Another important aspect is that the chemical structure of HS resembles that of organic contaminants. Consequently, soil microorganisms, and especially wood-decaying and litter-decomposing fungi, with the ability to degrade and even mineralize HS are adapted to degrade contaminants present in the soil (Kästner and Hofrichter, 2001; Steffen et al., 2002b). This adaptability to contaminants represents an advantage for soil decontamination by fungi.

1.2 Contamination of soil

Soil is highly resistant to perturbations due to its capacity to carry out functions by a set of "defense" mechanisms. Such mechanisms include sorption/desorption processes, biological or chemical reduction-oxidation reactions, hydrolysis, volatilization, or chelation. The mechanisms can be dramatically affected if the threats are continuous and/or exceed the soil's natural capacity to keep itself in balance. In 2006 the European Commission, in the Communication of Thematic Strategy for Soil Protection, identified eight threats to soil quality. These include sealing, erosion, loss of organic matter, decline of biodiversity, compaction, hydrogeological risks, salinisation and contamination (Commission of the European Communities, 2006a). According to the European Soil Bureau Network, soil sealing "is the loss of soil resources due to covering of land for housing, roads or other construction works", while the term soil compaction refers to the "deterioration of soil structure by mechanistic pressure, predominantly from agricultural practices" (Jones et al., 2005).

Of these threats, soil contamination represents a serious problem throughout Europe since, according to recent estimates, around 250,000 contaminated sites require urgent treatment, which will cost millions of euros, approx. 2.25 billion euros for the period 2005 - 2013 (European Environment Agency, 2007).

Industrial development and agricultural activities have caused the majority of soil contamination, which is defined as "the appearance of a hazardous substance at a concentration level that poses a risk to a potential receptor" (van-Camp et al., 2004). According to the European Directive 2004/35/EC, the concentration levels at which soil is considered to be contaminated are independently regulated by each European Member State (Commission of the European Communities, 2006b). As an example, the Finnish Government Decree on the Assessment of Soil Contamination and Remediation Needs establishes a threshold value for benzo[a]pyrene of 0.2 mg/kg from which a risk assessment must be initiated in the area (Ministry of the Environment, Finland, 2007). Since soil is not an isolated biotope, the contamination may be transported to the adjacent biotopes (e.g. groundwater, river) causing a serious threat to the ecosystem and to human health.

Soil contamination is classified as either localized or diffuse contamination (Jones et al., 2005). The sources of localized contamination are frequently related to industrial discharges, improper waste disposal or accidental spills during the transportation or handling of hazardous compounds. Diffuse contamination is produced mostly by intensive agriculture and forestry practices, transportation, as well as industrial activities leading to atmospheric deposition. The European Environment Agency has identified industrial and other commercial activities as well as the disposal and treatment of municipal waste as the most important sources of both localized and diffuse contamination of petroleum hydrocarbons and heavy metals in Europe (Table 1.1; European Environment Agency, 2007).

The compounds responsible for contamination (Table 1.1) are very diverse in terms of their chemical structure and properties but share the characteristic of being anthropogenic, usually recalcitrant and toxic to organisms living in the environment. In addition, several soil contaminants are related to cancer development, endocrine disruption, or teratogenesis (i.e. birth abnormalities) in humans. Soil and contaminant properties determine the fate of contamination in the soil as well as the interactions with soil constituents. Soil factors affecting contamination transfer are the organic matter content, the type of mineral constituents, structure, oxidation potential, and surface area. The most important contaminant properties are molecular mass, the oxidation state, solubility, molecular structure, the octanol-water partition coefficient and vapor pressure (Mirsal, 2008; Cardona García, 2009). For instance, when a soil rich in organic matter is contaminated with a non-ionic contaminant with large molecular mass and low octanol-water partition coefficient – the case of some high molecular weight polycyclic aromatic hydrocarbons − the contaminant will likely bind to humic substances and, consequently, leaching to the groundwater will probably not occur (Mueller et al., 1996). In the case of organic phosphorous insecticides (members of the phosphoric acid ester groups), the nature of the aliphatic cyclic group and the soil pH will influence the adsorption of the insecticide on clay surfaces. The ester bond of the molecule is stable at neutral or acid pH, but it is hydrolyzable under alkaline conditions (Mirsal, 2008). Due to the complexity of the soil system and the heterogeneity of the contamination, the prediction of the actual fate of the contaminants is not straightforward. Nevertheless, an adequate knowledge of soil properties will facilitate the best decontamination strategy.

Table 1.1 The most relevant contaminants in European soils and their origin.

Contaminants	Example of compounds	Origin of contamination[a]	Estimated percentage[b]	References
Heavy metals	Copper, zinc, cadmium, lead, mercury, chromium	Application of animal manure (D) Military facilities (L) Gasoline stations (L) Sawmills and wood preservation sites (L) Mining and metallurgical industry (L,D)	37.3	van-Camp et al., 2004; Mirsal, 2008
Petroleum hydrocarbons (aliphatic and cyclo hydrocarbons)	Alkanes, alkenes, cycloalkanes	Oil industry (L,D) Gasoline stations (L)	33.7	European Environment Agency, 2007
PAHs	benzo[a]pyrene, chrysene, fluoranthene	Oil industry (L,D) Gasoline stations (L) Manufactured gas plants (L,D) Wood preservation sites (L) Municipal waste incineration (L,D) Automobile exhaust (D)	13.3	Crawford and Crawford, 1996; Achten and Hofmann, 2009
Monomeric aromatic hydrocarbons	benzene, toluene, ethylbenzene, xylene	Oil industry (L,D) Gasoline stations (L) Manufactured gas plants (L,D)	6	Crawford and Crawford, 1996
Chlorinated compounds	PCP, PCBs, PCDD/Fs, Chlorinated solvents (trichloroethylene, methylne chloride)	Manufacture and use of pesticide and herbicide (D) Wood preservation sites (L) Pulp and paper production (L) Municipal waste incineration (L,D) Plastics, fire-retardants manufacture (L,D)	Chlorinated phenols - 3.6 Chlorinated hydrocarbons - 2.4	Crawford and Crawford, 1996; Merino et al., 2009
Nitroaromatics	TNT, Nitrobenzene, nitrophenols, atrazine	Manufacture of aniline, dyes, drugs (L,D) Explosive industry, military facilities (L,D) Manufacture of pesticides and herbicides (D)	[c]	Ye et al., 2004

[a] L = localized contamination; D = diffuse contamination.

[b] According to the European Environmental Agency, the estimated percentage is based on the frequency with which a specific contaminant is reported to be the most important in the investigated site (European Environment Agency, 2007).

[c] Information not available.

PAHs = polycyclic aromatic hydrocarbons; PCP = pentachlorophenol; PCBs = polychlorinated biphenyls; PCDD/Fs = dibenzo-*p*-dioxins and -furans; TNT = trinitrotoluene.

1.2.1 Contamination by polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are constituted of two or more fused benzene rings sharing a pair of carbon atoms between two adjacent rings in linear, cluster or angular arrangements. PAHs contain only carbon and hydrogen atoms. Petroleum-derived heterocyclic compounds may also contain sulphur (e.g., dibenzothiophene), nitrogen or oxygen atoms (Dabestani and Ivanov, 1999). Except for PAH compounds containing fjord regions (Fig. 1.1), PAHs have a planar geometry. The alternating single and double bonds give PAHs an unusual stability and, consequently, resistance to microbial degradation. The water solubility and bioavailability of PAHs is low, which decrease as the molecular mass increases (Baird and Cann, 2008; Table 1.2). Furthermore, PAHs with a bay (e.g., chrysene or benzo[a]pyrene) or fjord regions (e.g. benzo[c]phenanthrene or dibenzo[a,l]pyrene) in their molecular structure are the most potential carcinogens (Fig. 1.1 and Table 1.2). For instance, when entering the organism, benzo[a]pyrene is activated by a series of metabolic reactions that lead to the ultimate carcinogenic metabolite, a reactive diol epoxide, which may bind covalently to DNA, leading to mutations and, consequently, resulting in tumours (Mattsson, 2008; Table 1.2).

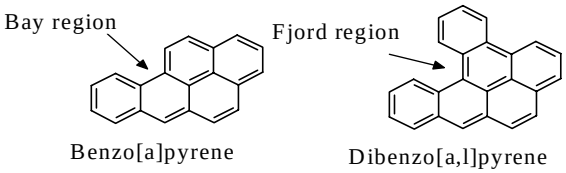
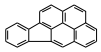
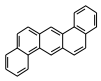


Figure 1.1 Bay and fjord regions in PAH molecular structure.

PAHs are commonly found in soil, even in remote areas without human settlements (Johnsen and Karlson, 2007). Natural inputs of PAHs occur during volcanic eruptions and forest fires. However, most PAHs originate from anthropogenic sources, such as the incomplete combustion of fossil fuels, wood and waste, automobile exhaust, and unintentional petroleum derivatives spills (Dabestani and Ivanov, 1999). Diffuse contamination occurs mainly via atmospheric deposition of PAHs adsorbed to particles. Wind transports these particles to further localizations and the PAHs adsorbed to particles are deposited directly onto the soil or indirectly through the vegetation. It is estimated that soil receives 0.7 - 1 mg/m^2 of PAHs annually by air emissions (Wilcke, 2000; Johnsen and Karlson, 2007). Accidental crude oil spills in the sea are important localized sources of PAH contamination. Due the low solubility of the aromatic fraction of crude oil, which accounts for 50% of all the fractions, PAHs are mostly deposited into the sediments or transported to shorelines and other marine ecosystems, such as coastal marshes or estuaries (Dabestani and Ivanov, 1999; AECIPE, 2002). Estimations predict that 1.7 - 8.8 x 10^6 tons of crude oil enter into coastal environments annually. As an example, recently the oil rig Deepwater Horizon exploded and sank in the Gulf of Mexico on the 22nd of April 2010 spreading up to 800 m^3 of light crude oil per day (CEDRE, 2010). Once in the soil, PAHs may be degraded or transformed, which will determine their transport, distribution and levels of concentration.

Table 1.2 Chemical structure and properties of the 16 EPA PAHs, listed according to their water solubility.

Compound	Structure	Water solubility (mg/l)[a]	Molecular mass (g/mol)[a]	Vapor pressure (Pa at 25°C)[a,b]	Ionization potential (eV)[a]	$logK_{ow}$[a,c]	IARC[d]	Concentration in soil[e]	
								Forest (µg/kg)	Urban (µg/kg)
Naphthalene		31.0	128.19	10.40	8.12 ± 0.02	3.37	2B	33	39
Acenaphthylene		16.1	152.20	0.90	8.22 ± 0.04	4.00	NA	3.4	16
Phenanthrene		4.57	178.23	0.020	7.90	3.24	3	60	190
Acenaphthene		3.80	152.21	0.30	7.68 ± 0.05	3.92	3	2	57
Fluorene		1.90	166.22	0.090	7.88 ± 0.05	4.18	3	6.9	23
Fluoranthene		0.26	202.26	0.00123	7.9 ± 0.1	5.22	3	118	805
Pyrene		0.132	202.26	0.0006	7.43 ± 0.01	5.18	3	72	593
Indeno [1,2,3-c,d]pyrene		0.062	276.33	[f]	[f]	[f]	2B	82	387
Anthracene		0.045	178.23	0.0010	7.44 ± 0.06	4.54	3	8.6	58
Benzo [a]anthracene		0.011	228.29	2.8×10^{-5}	7.53 ± 0.30	5.91	2B	43	437
Benzo [a]pyrene		0.0038	252.31	7.0×10^{-7}	7.10	6.04	1	39	350
Benzo [b]fluoranthene		0.0015	252.31	6.7×10^{-5} (at 20°C)	7.70	5.80	2B	158	456
Benzo [k]fluoranthene		0.0008	252.31	5.2×10^{-8}	[f]	6.00	2B	186	236
Chrysene		0.0006	228.29	5.7×10^{-7}	7.60 ± 0.03	1,65	2B	117	278
Dibenzo [a,h]anthracene		0.0006	278.35	3.7×10^{-10}	7.38 ± 0.02	[f]	2A	15	55
Benzo [g,h,i]perylene		0.00026	268.35	1.3×10^{-8}	7.31	6.50	3	62	370

[a] Dabestani and Ivanov, 1999; Steffen, 2003; U.S. National Library of Medicine, 2010.
[b] U.S. National Library of Medicine, 2010.
[c] $logK_{ow}$ is the logarithm for the octanol-water partition coefficient of a specific compound.
[d] IARC is the International Agency for Research on Cancer that classified compounds with carcinogenic risk as: 1-carcinogenic to humans; 2A-probably carcinogenic to humans; 2B-possibly carcinogenic to humans; 3-not classifiable as to its carcinogenicity to humans; 4-probably not carcinogenic to humans; NA-not classified (IARC, 2010).
[e] Values show PAH concentration in forest or temperate urban soil (Wilcke, 2000).
[f] data not available.

Soil organic matter is the most important factor affecting the fate of PAHs in soil. Sorption of PAHs takes place mainly on the surface of SOM and, in some cases, in the internal binding sites of the organic molecules. The binding affinity of PAHs to SOM is explained by the organic carbon (OC) partition coefficient (K_{oc}), which is described by the partition coefficient (K) normalized against the organic fraction of the soil (f_{oc}; Eq. 1.1) (Wilcke, 2000).

$$\text{Eq. 1.1:}\quad K_{oc} = \frac{K}{f_{oc}}$$

The logarithm for K_{oc} may also be estimated with the logarithm for the octanol-water partition coefficient ($\log K_{ow}$) using different equations, as reviewed by Wilcke (2000) (e.g., Eq. 1.2 and 1.3). In general, $\log K_{ow}$ increases with PAH molecular mass (Table 1.2), which means more affinity for organic matter and, consequently, lower bioavailability for microbial degradation.

$$\text{Eq. 1.2:}\quad \log K_{oc} = \log K_{ow} - 0.317$$

$$\text{Eq. 1.3:}\quad \log K_{oc} = 0.989 \times \log K_{ow}$$

Even though PAHs are quite resistant to microbial degradation, 2-, 3- and 4-ring PAHs are more susceptible to microbial attack (Johnsen and Karlson, 2007). Oxidation of low molecular weight PAHs may occur by bacteria in the genus of *Pseudomonas* and *Rhodococcus* via dioxygenase enzymes, forming *cis*-dihydrodiols, which can be further degraded to carbon dioxide and water via catechol formation (Fig. 1.2). The cytochrome P450 enzymes of *Mycobacterium* sp. or *Zygomycetous* fungi degrade PAHs to arene oxide and then to *trans*-dihydrodiols by the action epoxide hydrolase (Mueller et al., 1996; Bamforth and Singleton, 2005). High molecular mass PAHs can be degraded by wood-degrading fungi (white-rot and litter-decomposing fungi) due to their extracellular production of oxidative non-specific enzymes (Gramss et al., 1999b; Cerniglia and Sutherland, 2001; Pointing, 2001; Steffen et al., 2002a). The metabolites can undergo successive degradation by bacteria. The degradation of PAHs by wood-degrading fungi is discussed in more detail in section 1.5.1.

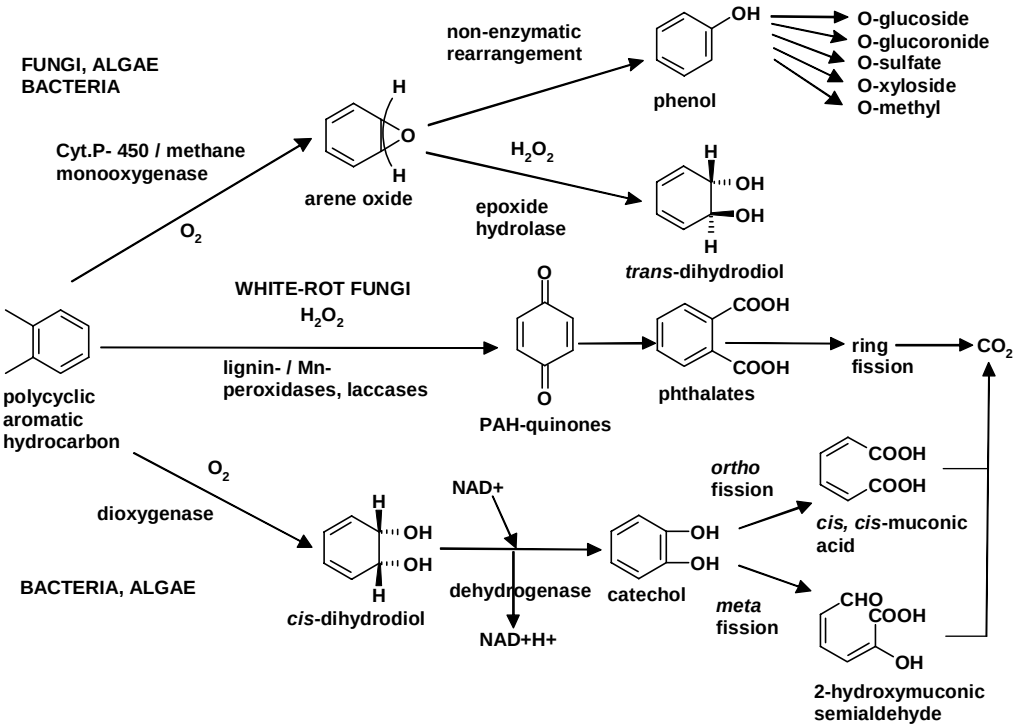


Figure 1.2 Degradation pathways of polycyclic aromatic hydrocarbons (PAHs) by bacteria and fungi (After Cerniglia and Sutherland, 2001). Figure courtesy of Kari Steffen.

1.2.2 Contamination by wood preservation compounds

Chlorophenols, creosote and copper-chromium-arsenic (CCA) preservatives have been used or are still in use in many sawmills and wood-preservation facilities to protect timber against microbial attack. In Finland, around 23,400 tons of chlorophenols wood preservatives were produced and used between 1940 and 1984 with the commercial name Ky-5 (Kitunen, 1990). In the late 1980s, nearly 300 Finnish sawmill sites were contaminated with chlorophenols and other chlorinated compounds originating from the Ky-5 product, namely polychlorinated phenoxyphenols (PCPPs) and polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) (Kitunen et al., 1985). PCDD/Fs accumulated mostly on the surface of the soil, while chlorophenols either migrated to the vadose zone and groundwater or were degraded. (Kitunen et al., 1987). Nowadays, there are nearly 450 potentially contaminated sawmill sites and approximately 100 sites requiring remediation in the near future (Haavisto, T. Finnish Environment Institute, personal communication). If landfilling and stabilization are excluded, the only method that really solves the problem of highly contaminated sawmill sites is excavation and combustion at high temperatures (over 1300 ºC; Jaakkonen, S. Finnish Environment Institute, personal communication). However, sawmill soil frequently has a high organic matter content as a result of the remaining wood particles. When thermal desorption is applied as a combustion method, several drawbacks arise due to the high organic matter content. The process capacity is reduced because desorption needs more time. There is a risk that the heat exchanger capacity is not sufficient to cool down the exhaust gas because of its high calorific content, which may damage the filter unit. Additionally, the higher

exhaust gas flow rate may block the filter unit. Thus, more fuel is needed, raising the cost of the treatment (Rantsi, R. Niska ja Nyyssönen Oy, and Tunturi, M., Ekokem-Palvelu Oy, personal communications). Occasionally, soil that has a high organic content is treated with incinerators specifically designed for it (Tunturi, M., Ekokem-Palvelu Oy, personal communication). But, in general, all combustion methods are very expensive and only suitable for hot spots. Composting of chlorophenols contaminated soil has also been applied (Laine, 1998). While the chlorophenols were effectively degraded, PCDD/Fs remained almost intact or even increased after 25 weeks of composting (Laine and Jørgensen, 1997). Overall, soil contaminated by wood preservation products and other compounds still represents a problem in Finland since the majority of soils are deposited in landfills or, exceptionally, combusted. An adequate technology for treating soils in a more environmentally friendly and cost-effective manner is still unavailable.

1.3 Bioremediation

Bioremediation, "the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state" (Bamforth and Singleton, 2005), offers an alternative to physical and chemical treatments of contaminated soil. Generally, bioremediation refers to the microorganisms (bacteria, archaea, fungi or algae) as the contaminant degraders, while the more specific term phytoremediation is often applied to the use of plants (Crawford and Crawford, 1996). Bioremediation technologies can be classified into two categories: *ex situ* and *in situ*. Both treatments may be applied in many different modes, either by adding nutrients, usually nitrogen and phosphorous, and/or oxygen to stimulate autochthonous microbial degradation (biostimulation) or by introducing exogenous microorganisms to enhance the degradation process (bioaugmentation; Scullion, 2006).

For cases in which contaminants are available and well localized, *in situ* techniques would be the least disruptive methods for the environment, as excavation is avoided and soil properties and functions are maintained (Mirsal, 2008). Recently, attempts have arisen to promote monitored natural attenuation (MNA), which refers to the management of "the observed reduction of contaminant concentration" (Rügner et al., 2006).

Ex situ techniques involve excavating the contaminated soil and transporting it to specific facilities for the treatment process. If soil is treated on the contaminated site, the bioremediation mode is then called *on site*. Both *ex situ* and *on site* treatments are mostly applied when the contaminant is recalcitrant and the concentration high. Moreover, if soil permeability is low and/or organic matter is high, *ex situ* technologies are preferred. Finally, another criterion for selecting *ex situ* remediation may be when the climate conditions hinder *in situ* treatments, or when authorities require the treatment be accomplished rapidly (Robles-González et al., 2008). Solid-phase technologies such as biopiling, composting and slurry-phase bioreactors are examples of *ex situ* practices (Crawford and Crawford, 1996).

The success of any bioremediation technique depends on several soil factors, such as temperature, pH, oxygen, nutrient concentration, water content, and contaminant bioavailability (Scullion, 2006). Bioavailability is defined as the fraction of a contaminant that is susceptible to being degraded by microbes. Contaminant bioavailability may be predicted with sequential supercritical fluid extraction (SFE) of the target contaminant to obtain desorption curves of the bound fraction. SFE gives the fraction that is rapidly desorbed from the soil, the actual bioavailable fraction, and the fraction that remains in the soil (Cajthaml and Šašek, 2005; Leonardi et al., 2007). The characterization of soil properties, the extent of contamination, the microbial population, and the potential toxicity of degradation products are also key factors in the decision-making for the best available bioremediation technology (Admassu and Korus, 1996; Scullion, 2006).

1.3.1 Slurry-phase bioreactor

If the contaminant is absorbed by soil particles and, consequently, the bioavailability becomes the limiting factor, a slurry-phase bioreactor may be a good option. Large molecular weight contaminants will be efficiently degraded by increasing the mass transfer from the solid phase to the aqueous phase where the degradation is assumed to occur (Admassu and Korus, 1996). In a slurry reactor, the agitation and the aeration increase the mass transfer rate and enhance the contact between the microorganisms and solid particles (Doelman and Breedveld, 1999). An good homogenization, together with sufficient aeration, are achieved when the slurry contains 10 - 30% of the soil (w/v) being previously crushed or fractionated into fine particles of 500 - 800 μm (Robles-González et al., 2008). The bioreactor may be inoculated with an enriched microbial consortium from the contaminated soil or with exogenous specialized microorganism(s): bacteria or fungi. The higher efficiency of slurry bioreactors compared to other *ex situ* treatments is attributed to the control of pH, temperature, dissolved oxygen and the supply of nutrients. In some cases, surfactants or solvents may be applied in order to facilitate contaminant desorption. Bioreactors can also operate under anoxic conditions by adding nitrate or sulphate as electron acceptors. Anaerobic bioreactors are less common (Robles-González et al., 2008). A schematic configuration of a slurry-phase bioreactor is shown in Fig. 1.3.

Generally, slurry-phase bioreactors have been applied to degrade petroleum hydrocarbons (Machín-Ramírez et al., 2008) and to treat PAH-contaminated soil or sediments (Villemur et al., 2000; Saponaro et al., 2002; Abbondanzi et al., 2006). Also, halogenated compounds such as polychlorinated biphenyls (PCBs) or nitroaromatic compounds (e.g., 2,4,6-trinitrotoluene, TNT) have efficiently been degraded by slurry bioreactors (Fuller and Manning, 2004; Di Toro et al., 2006; Robles-González et al., 2008).

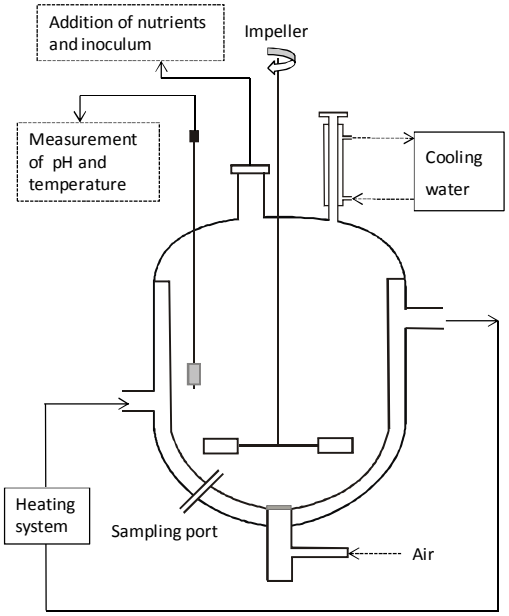


Figure 1.3 Schematic representation of the slurry-phase bioreactor used in this study. Modified from Quintero et al. (2007).

Commercial application exists mainly for aerobic slurry reactors with dimensions varying between 3 and 25 m in diameter and 4.5 - 8 m in height (Robles-González et al., 2008). Besides their clear advantages - more efficient, less land area requirement, and bioavailability improvement -, slurry-phase bioreactors have an important drawback: the high cost derived from soil excavation and handling, bioreactor construction and operation, and large energy and water consumption. Nevertheless, less expensive reactors consisting of lagoons of 24 m x 15 m are also applied at full scale (Robles-González et al., 2008). Due to the treatment cost of slurry reactors, more cost-efficient *ex situ* technologies, such as solid-phase treatments, are usually applied.

1.3.2 Solid-phase treatment

Ex situ solid-phase treatment can be performed in biopiles or windrows. Biopiles refer to the piling of the contaminated soil above an impermeable membrane. The typical size of a biopile is 3 - 6 m in height, 3 - 4 m in width and 10 - 20 m in length. Aeration is supplied by perforated tubes set on the bottom or at different levels. Windrows, in contrast, are aerated by the frequent turning or tilling of piles (Vik and Bardos, 2002; Escolano Segovia, 2009). For this purpose, the height of a windrow (1 - 2 m) is lower than that of a biopile. Both biopiles and windrows may be improved by nutrient amendments and/or the addition of lignocellulosic materials (e.g., straw, bark, wood chips) to facilitate oxygen diffusion and thus enhance microbial degradation. According to the Contaminated Land and Rehabilitation Network for Environmental Technologies (Vik and Bardos, 2002), when some lignocellulosic material is added the technology is called composting. Bioaugmentation with indigenous microbes is also possible (Jørgensen et al., 2000; Vik and Bardos, 2002; Escolano Segovia, 2009). A schematic configuration of a biopile is shown in Fig. 1.4.

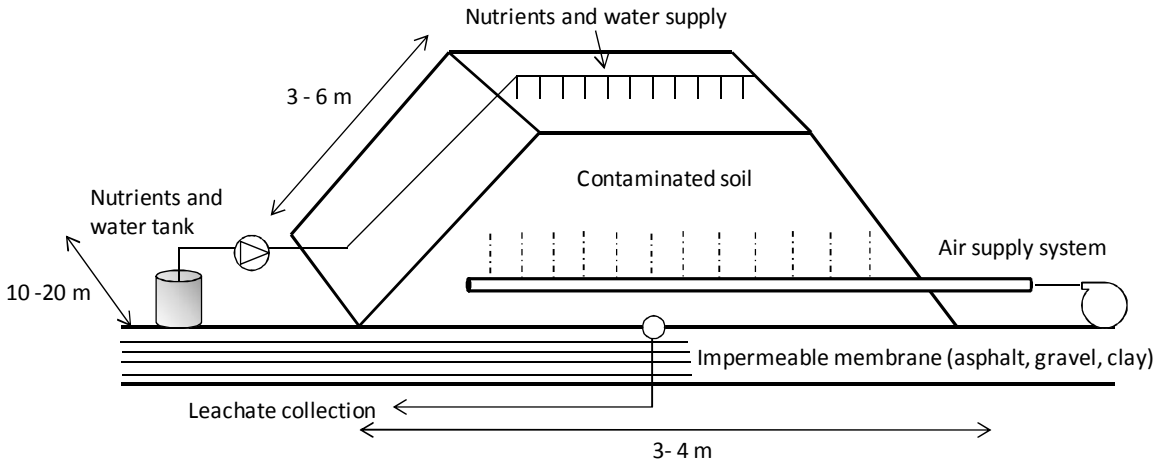


Figure 1.4 Schematic representation of a biopile (not to scale). Modified from Toffoletto et al. (2005).

Soil contaminated with petroleum hydrocarbons have been treated in pilot or semi-field scale biopiles. Without large investments, 70 - 95% of petroleum hydrocarbons have been successfully removed by biopiling (Rojas-Avelizapa et al., 2007; Lin et al., 2010) or composting the contaminated soil during one- to five-month treatment (Jørgensen et al., 2000). Less frequent, but equally successful, have been biopiles to treat chlorophenols (90% degradation after six months; Laine and Jørgensen, 1997) and nitroaromatic compounds (around 82% after six months; Lewis et al., 2004; Clark and Boopathy, 2007). Despite the relatively long treatment time required, solid-phase treatment is a cost-effective alternative for *ex situ* bioremediation due to the high degradation performance achieved.

When one or more of the following factors are present, a solid-phase treatment may be an ideal option: i) if the soil is poor in organic matter and porosity, the addition of natural lignocellulosic amendments as bulking agents (i.e., composting) may be enough to promote biodegradation (Laine and Jørgensen, 1996; Rhykerd et al., 1999); ii) if the soil is contaminated with low molecular mass compounds (Sims et al., 1999) and/or iii) if alternative treatments are more expensive or when rapid decontamination is not required.

1.4 Wood-degrading fungi

Fungi with contaminant-degrading capabilities are mostly wood-degrading basidiomycetes, which form macroscopic fruiting bodies (Fig. 1.5). These fungi are primarily found in the order *Agaricales* (e.g., *Stropharia rugosoannulata*, *Agrocybe praecox*, *Pleurotus ostreatus*) and *Polyporales* (e.g., *Phanerochaete chysosporium*, *Trametes versicolor*, *Bjerkandera adusta*, *Irpex lacteus*; Dix and Webster, 1995; Steffen et al., 2002a; Hibbett et al., 2007; Steffen et al., 2007). In addition, several fungi belonging to the phyla *Ascomycota* (Carvalho et al., 2009), *Zygomycota* (e.g., *Cunninghamella elegans*) and anamorphic ascomycetes (e.g., *Aspergillus* spp., *Penicillium* spp., *Paecilomyces* spp.) have also demonstrated the ability to degrade contaminants (Gesell et al., 2004; Tortella et al., 2005).

Figure 1.5 Fruiting body of some wood-degrading fungi applied in bioremediation and in this thesis. From left to right: *Agrocybe praecox, Bjerkandera adusta* and *Stropharia rugosoannulata*. Photos courtesy of Roger Phillips[1].

For bioremediation applications, it is preferable that fungi are in the vegetative phase of the life cycle when the mycelium is building up and can penetrate into the soil. Likewise, it is during mycelium expansion when the extracellular enzymes involved in contaminant degradation are produced (Dix and Webster, 1995). The main ecological characteristic of contaminant-degrading fungi is the saprotrophy, meaning that they obtain nutrients and energy by decomposing dead biomass. Accordingly, these fungi can be classified as wood-decaying fungi *sensu stricto* and litter-decomposing fungi (Steffen, 2003).

Wood-decaying fungi colonize standing or fallen wood, such as branches, stumps, and trunks producing either white- or brown-rot. Brown-rot fungi (BRF), which are associated mainly with softwood decay, depolymerize cellulose and hemicellulose, leaving lignin almost intact and thus causing a crumbly and brownish cubical-shaped wood (Eriksson et al., 1990; Hatakka, 2001; Martínez et al., 2005). Due to the inability of BRF to produce lignin-modifying enzymes, they are generally not applied in bioremediation (Eriksson et al., 1990). White-rot fungi (WRF) are the only organisms, together with some litter-decomposing fungi, capable to degrade extensively lignin. Some WRF simultaneously decay all of the components of the wood, while others, referred to as selective WRF, degrade preferably lignin and hemicellulose without substantially degrading cellulose. Typically, white rotten wood has a fibrous and light appearance (Hatakka, 2001; Martínez et al., 2005). WRF are more common on the hardwoods of angiosperm trees, but some species also grow on softwood, such as *Phellinus chrysoloma* in spruce and *Heterobasidion* sp., a tree pathogen, in pine and spruce (Dix and Webster, 1995; Carlile et al., 2001; Martínez et al., 2005; Niemelä, 2005).

[1] http://www.rogersmushrooms.com/

Soil-litter comprises fallen leaves, small branches, needles and all type of lignocellulosic forest debris deposited on the upper layer of forest soil. A wide diversity of microbes participate in the decay of soil-litter, but the most active decomposers are basidiomycetes fungi, which constitute more than 60% of the total microbial biomass in soil-litter (Dix and Webster, 1995). As WRF, litter-decomposing fungi (LDF) have the ability to decompose lignin and polysaccharides causing white rot in soil-litter (Dix and Webster, 1995; Steffen, 2003; Osono, 2007). There are also fungi (e.g., *Hypholoma* spp.) whose habitat overlaps with those of WRF and LDF, and which are capable of colonizing soil from the base of wood debris (Steffen, 2003).

1.4.1 Lignocellulosic substrates

Lignocellulosic material is essential for the growth of fungi in nature and also crucial in bioremediation, since contaminated soil is generally poor in nutrients and the soil itself is a hostile environment for some fungal species (Baldrian, 2008b). The lignocellulosic substrates used in fungal bioremediation comprise different residues from agriculture (e.g., wheat straw, sugar cane bagasse, corn cobs) and forestry (e.g., sawdust, wood chips, bark; Bennett et al., 2001; Sánchez, 2009), as well as from the food industry (e.g., spent mushroom compost; Lau et al., 2003). Substrate formulation is one of the main factors for a successful fungal bioremediation application (Leštan et al., 1996). Generally, the preparation of fungal lignocellulosic inocula is preceded by liquid cultivation. Once fungi have grown sufficiently in the substrate, the inoculum is introduced into the soil. Such a substrate serves as a carbon and energy source and supporting material for fungal hyphae during the bioremediation process. There are several strategies for introducing the substrate into to soil: placing it on the surface, within the soil layers, or mixed or embedded in a specific carrier substance commonly composed of some wood residue which acts as a substrate itself (Leštan and Lamar, 1996; Leštan et al., 1996; Eggen, 1999; Bennett et al., 2001; Ford et al., 2007a; Ford et al., 2007b). It is desirable that the substrate is resistant to colonization by endogenous soil microbes (Leštan et al., 1996). However, most of the commonly applied substrates used for the introduction of fungi into the soil, may easily be colonized by other fungal species (generally anamorphic ascomycetes, i.e., moulds) mainly because of the high content of cellulose (e.g., 45% in corn cobs; Sánchez, 2009). Consequently, colonization by wood-degrading fungi may be hindered.

1.4.2 Bark as substrate for fungal growth and inocula

Bark, the outermost layer of the tree trunk, is considered an undesirable material for the pulp and paper industry due to its small amount of usable fibres and the content of its extractives (Biermann, 1993). Despite its apparent lack of industrial applications, bark is used for the production of adhesives and dyes, or as food additive, due to its antioxidant properties (Biermann, 1993; Karonen et al., 2004a). Bark is also an amendment for composting formulations (Cunha-Queda et al., 2007) or is burned as fuel.

In the field of fungal bioremediation, bark has a potential application as a lignocellulosic substrate as it is relatively inexpensive and widely available. Furthermore, the composition and functional role (i.e.protection against microbial or insect attacks and reserve of nutrients; Sjöström, 1993) of its extractives have an advantage for the fungi. Bark extractives provide a constant carbon supply and prevent a rapid colonization by competing microbes. In fact, Steffen et al. (2007) showed that litter-decomposing fungi grew extensively in pine bark and that no contamination by undesired microbes occurred during the bioremediation process. Information regarding the chemical composition of Scots pine bark extractives is only available from several phenolic compounds, such as lignans, catechins, flavonoids and procyanidins (Pan and Lundgren, 1996; Karonen et al., 2004a; Karonen et al., 2004b; Sinkkonen et al., 2005; Sinkkonen et al., 2006), but the composition of more lipophilic extractives is only known for the corresponding wood (Dorado et al., 2000; Martínez-Íñigo et al., 2000; Dorado et al., 2001; Willför et al., 2003). Moreover, no study has yet addressed the degradation of pine bark by basidiomycetes. New knowledge on the use of Scots pine bark for bioremediation and a description of its chemical composition and degradation has been obtained from this thesis.

As in the corresponding wood, bark is also composed of the three main natural biopolymers: cellulose, hemicellulose and lignin, but their contents vary among different plant species. Lignin is an amorphous, three-dimensional and hydrophobic polymer which gives plants their rigidity and strength (Sjöström, 1993; Argyropoulos and Menachem, 1997; Davin et al., 2009). Lignin also protects plants against microbial attack and other environmental threats (Eriksson et al., 1990). Three types of phenyl-propanoid units are the precursors of the lignin structure: *p*-coumaryl, coniferyl and sinapyl alcohols (Eaton and Hale, 1993). After polymerization, the respective lignin types are called *p*-hydroxyphenyl, guaiacyl and syringyl (Higuchi, 2006). Comparison of the lignin content in bark between several different tree species is restricted because of the extraction procedure. In general, the lignin content of softwood barks (e.g., 45%, w/w, in *Pinus sylvestris* and 33%, w/w, in *Pinus taeda*) and hardwood barks (e.g., 43% in *Fagus sylvatica*) is similar but higher than the polysaccharides content (Fengel and Wegener, 1989a; Kostov et al., 1991; Fradinho et al., 2002).

Cellulose is a linear homopolysaccharide of β-D-glucopyranose units linked by 1→4 glycosidic bonds. The main unit of cellulose is the disaccharide cellobiose, which forms a linear polymer with other cellobiose units to form microfibrils, fibrils and, additionally, cellulose fibres. Crystalline regions, which are more resistant to microbial degradation, alternate with amorphous regions in the cellulose fibers (Eriksson et al., 1990; Sjöström, 1993). In contrast to cellulose, hemicellulose is an amorphous molecule with a branched or linear configuration and composed of several monomeric sugars and sugar acids, namely glucose, mannose, galactose, xylose, arabinose, minor amounts of rhamnose, glucuronic acid, 4-*O*-methylglucuronic acid and galacturonic acid. Hemicelluloses are located around cellulose microfibrils occupying the spaces between fibrils and are covalently bound to lignin functioning as supporting material to cell walls (Eriksson et al., 1990; Sjöström,

1993). The content of polysaccharides in bark varies among different tree species, but in general it is dominated by the cellulose content, which is in the range of 20 - 33% (w/w; Fengel and Wegener, 1989a). The content of different sugars is also specie specific, but normally glucose is the most abundant, followed by mannose and xylose (Fengel and Wegener, 1989a; Kostov et al., 1991; Sjöström, 1993; Fradinho et al., 2002). For example, in Scots pine bark the glucose content is 30% while the xylose and mannose content is only 5.8% and 4.5%, respectively (Fengel and Wegener, 1989a).

1.4.3 Lignocellulose-degrading enzymes

The mechanism of contaminant degradation by fungi (wood- and litter-decomposing fungi) is based on the production of the oxidoreductases and hydrolytic enzymes involved in the degradation of lignin and polysaccharides, respectively.

1.4.3.1 Lignin modifying enzymes

Fungal oxidoreductase laccase (EC 1.10.3.2) and the peroxidases lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.13), and versatile peroxidase (VP; EC 1.11.1.16)), a hybrid form of MnP and LiP, are responsible for the degradation of lignin (Hatakka, 2001; Hofrichter, 2002; Martínez et al., 2005; Baldrian, 2006). In addition, other enzymes are indirectly involved in lignin modification. For example, the hydrogen peroxide-generating enzymes glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) are essential in the catalytic cycle of peroxidases since they require H_2O_2 as an electron acceptor (Hatakka, 2001; Lundell et al., 2010). Moreover, cellobiose-oxidizing enzymes, that is to say, cellobiose dehydrogenase and cellobiose:quinone oxidoreductase, are also proposed to be involved in the degradation of non-phenolic substructures of lignin by the formation of reactive hydroxyl radicals $^{\bullet}OH$ (Hildén et al., 2000).

Laccases are mostly extracellular multicopper oxidases, although intracellular laccases have also been detected in wood-decaying fungi. Fungal laccases are glycoproteins of 60 - 70 kDa which catalyze the oxidation of the phenolic substructures of lignin via one molecular oxygen reduction to water (Hatakka, 2001; Baldrian, 2006). Other non-phenolic compounds with high redox potential, including PAHs or other recalcitrant compounds, may also be oxidized by laccase in the presence of either natural mediators derived from oxidized lignin (i.e. *p*-coumaric acid or syringaldehyde; Camarero et al., 2005) or synthetic ones [i.e. ABTS (2,2´-azinobis(3-ethylthiazoline-6-sulfonate)) or 1-hydroxybenzotriazole (HBT); reviewed by Baldrian, 2006]. Laccase has low redox potential (450 - 800 mV) and typical isoelectric points ranging from 3.0 to 7.0. Basidiomycetes, ascomycetes and anamorphic ascomycetes are reported to produce various isoforms of laccases (Baldrian, 2006). Although WRF in general are the most active laccase producers, it has been discovered that the most studied lignin-degrading fungus, *Phanerochaete chrysosporium*, lacks laccase genes (Martínez et al., 2004). Consequently, the actual involvement of

laccase in lignin degradation is currently under investigation (Hatakka and Hammel, 2010; Lundell et al., 2010).

Manganese peroxidases (MnP) are heme-containing glycoproteins of 38 to 62 kDa which catalyze the oxidation of Mn^{2+}, ubiquitous in wood and soil, to Mn^{3+} using H_2O_2 as an electron acceptor (Hofrichter, 2002). Mn^{3+} is a strong oxidant and, after chelation and stabilization with a carboxylic acid such as oxalic or malic acid, it is able to oxidize phenolic and aromatic amines to phenoxyl and amino radicals, respectively (Wariishi et al., 1992; Kuan and Tien, 1993). The catalytic cycle of MnP involves two oxidative states of the enzyme (Hofrichter, 2002; Fig. 1.6): compound I (MnP_{oxid} I) and compound II (MnP_{oxid} II). First, H_2O_2 binds to the native enzyme forming the MnP_{oxid} I. Reduction of MnP_{oxid} I to MnP_{oxid} II, and of MnP_{oxid} II to the native enzyme occurs via one-electron oxidation of Mn^{2+} to Mn^{3+}. In contrast to laccase, MnP has a higher redox potential (> 1.0 V) and rather acidic isoelectric points (3.0 - 4.0), even though near-neutral MnPs have been described in litter-decomposing fungi (Steffen et al., 2002c). The inability of MnP to attack non-phenolic lignin moieties is overcome by several mechanisms. For example, Kapich et al. (1999b) proposed that lipid peroxidation, the oxidation of unsaturated fatty acids forming peroxyl radical, acts as a degradation mechanism of non-phenolic substructures by breaking the C_α-C_β and β-aryl ether bonds. MnP production is limited to basidiomycetes and the majority of WRF and LDF can secrete MnP (Hofrichter, 2002).

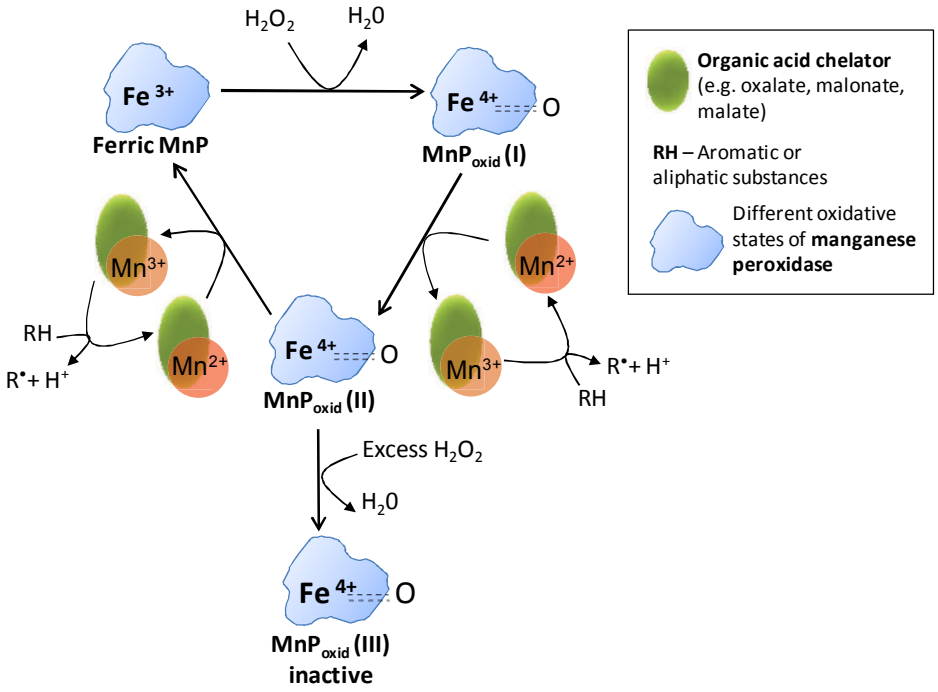


Figure 1.6 The catalytic cycle of manganese peroxidase (MnP), drawn according to that proposed by Hofrichter (2002).

Lignin peroxidase (LiP) is less common than MnP or laccase among WRF, and yet no LDF have been found to produce LiP (Steffen, 2003; Ruíz-Dueñas and Martínez, 2009). However, LiP, a glycosylated heme-containing peroxidase with 40 kDa and acidic

isoelectric points (2.5 - 3.0), is the only enzyme capable of oxidizing phenolic and non-phenolic rings via the reduction of H_2O_2 yielding aryl cation radicals and following further non-enzymatic reactions (Hatakka, 2001; Hammel and Cullen, 2008). Veratryl alcohol is a typical substrate of LiP, which in turn may act as a diffusible mediator oxidizing β-O-4 lignin dimer or non-accesible lignin substructures (Hatakka, 2001; Hammel and Cullen, 2008).

In addition, a hybrid enzyme possessing the catalytic properties of LiP and MnP, namely versatile peroxidase (VP), is also a lignin-modifying enzyme (Camarero et al., 1999). Unlike MnP and LiP, VP oxidizes both low and high redox potential compounds with or without Mn^{3+} mediation (Ruíz-Dueñas and Martínez, 2009). The versatility to degrade directly a wide variety of substrates, which LiP or MnP have enabled, makes VP an enzyme with a large potential for industrial applications including in the field of contaminant degradation (Pozdnyakova et al., 2010). VP has only been found in *Bjerkandera* and *Pleurotus* species (Hammel and Cullen, 2008; Ruíz-Dueñas and Martínez, 2009).

The fourth fungal-secreted heme peroxidase is the extracellular peroxidase from the ink cap fungus *Coprinus cinerea* (CiP). Unlike MnP and VP, CiP lacks the Mn^{2+} binding site. The ability of CiP to oxidize phenolic compounds might be exploited for the removal of phenols from waste waters (Qayyum et al., 2009; Hofrichter et al., 2010).

1.4.3.2 Cellulose and hemicellulose-degrading enzymes

Wood-degrading basidiomycetes have several endo- and exo-cleaving enzymes which attack cellulose. Both wood-decaying and litter-decomposing fungi show endo-1,4-β-glucanase (EC 3.2.1.4, endocellulase) activity, an endo-cleaving cellulolytic enzyme acting on the amorphous regions of the cellulose and randomly attacking the cellulose chain. On the contrary, the exo-cleaving enzyme cellobiohydrolase (EC 3.2.1.91; exocellulase) acts on the reducing or non-reducing ends of the chain and is typically active in crystalline cellulose. The resulting cellobiose is further metabolized by extracellular or intracellular β-glucosidase (EC 3.2.1.21) or dehydrogenated by cellobiose dehydrogenase (EC 1.1.99.18). Likewise, other non-enzymatic mechanisms, based on the Fenton reaction (Eq. 1.4), are also involved in the decomposition of cellulose by sapotrophic basidiomycetes, especially brown-rot fungi (Baldrian, 2008a).

$$\text{Eq. 1.4:} \quad H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\bullet}OH$$

Fungi degrade hemicellulose by producing a wide range of hemicellulases, depending on the type of hemicellulose they act upon. Xylanases degrade hemicelluloses with the main sugar unit xylan (e.g., glucuronoxylans and arabinoglucuronoxylan), and mannanases attack hemicelluloses with mannose as the main monosaccharide (e.g., glucomannans and galactoglucomannans). The most studied xylanases are the endo-1,4-β-xylanase (EC

3.2.1.8) and the β-D-xylosidase (EC 3.2.1.37). The former acts on glycosidic bonds in the xylan backbone, while the latter hydrolyzes xylooligosaccharides (Polizeli et al., 2005). The major fungal mannanases are the endo-β-1,4-mannanase (EC 3.2.1.78) and the exo-β-1,4-mannosidase (EC 3.2.1.25). Additionally, other enzymes, such as α-galactosidase (EC 3.2.1.22) and acetyl xylan esterases, together with the cellulase β-glucosidase, are involved in the breakdown of the hemicellulose chain (Tenkanen, 1998; Dhawan and Kaur, 2007). The litter composition determine the level and the type of of hydrolytic enzymes that LDF produce. As an example, Valášková et al. (2007) showed that endo-1,4-β-xylanase and endo-1,4-β-glucanase were the predominant enzymes when LDF were cultivated in oak (*Quercus petraea*) litter.

1.5 Fungi in bioremediation

The first time that fungi were proposed as specific contaminant degraders was in 1973 when Cerniglia and collaborators (Cerniglia and Perry, 1973) published a study on the potential of the non-ligninolytic fungus *Cunninghamella elegans* to degrade crude oil. One decade later, the same authors concluded that *C. elegans* used a similar mechanism as mammals to metabolize PAHs, which involved the intracellular enzymes cytochrome P450 monoooxygenase and epoxide hydrolase and yielded the formation of *trans*-dihydrodiols, phenols, quinones, and dihydrodiol-epoxides (reviewed by Cerniglia, 1997; Fig. 1.2).

The ability to degrade not only PAHs but also other recalcitrant pollutants was extended later to the white-rot fungus *Phanerochaete chrysosporium* (Bumpus et al., 1985). From that moment on, a considerable number of studies have been published on the potential of other WRF to degrade a wide range of contaminants (Table 1.3). The most studied fungi in addition to *P. chrysosporium* are *Trametes versicolor* (Logan et al., 1994; Johannes et al., 1996; Novotný et al., 1997; Majcherczyk et al., 1998; Tuomela et al., 1999), *Pleurotus ostreatus* (Bezalel et al., 1996a; Novotný et al., 1997; Beaudette et al., 1998), *Bjerkandera adusta* (Field et al., 1992; Kotterman et al., 1994; Beaudette et al., 1998), *Irpex lacteus* (reviewed by Novotný et al., 2009) and *Phlebia* spp. (van Aken et al., 1999; Mori and Kondo, 2002a; Mori and Kondo, 2002b; Mori et al., 2003; Kamei et al., 2005; Kamei et al., 2009). All of these studies linked the degradation of contaminants to the production of lignin-modifying enzymes (LMEs; Field et al., 1992; Sack and Gunther, 1993). Later, several studies extended the fungal degradation capability of PAHs (Gramss et al., 1999a; Steffen et al., 2002a; Steffen et al., 2003), TNT (Scheibner et al., 1997a) and dyes (Baldrian and Šnajdr, 2006) to litter-decomposing fungi, which mainly oxidize contaminants using MnP or laccase. Despite their potential, little is known about the degradation of other contaminants by LDF (Table 1.3).

Table 1.3 The most studied fungal species for bioremediation and their enzymes involved in the degradation of contaminants.

Fungus (ecophysiological group)[a]	Order (Family)[c]	Contaminant[d]	Lignin modifying enzymes	References
Agrocybe praecox (LDF)	Agaricales (Strophariaceae)	PAHs, TNT	Lacc., MnP	Scheibner et al., 1997a; Gramss et al., 1999a; Steffen et al., 2000; Steffen et al., 2002a.
Bjerkandera adusta (WRF)	Polyporales	PAHs, PCBs	(LiP)[e], MnP, VP	Field et al., 1992; Kotterman et al., 1994; Beaudette et al., 1998; Kotterman et al., 1998.
Irpex lacteus (WRF)	Polyporales	Dyes, PAHs, lindane, TNT, bisphenol A, nonylphenol, dimethyl phthalate	Lacc., LiP, MnP, VP	reviewed by Novotný et al., 2009.
Phanerochaete chrysosporium (WRF)	Polyporales	Synthetic dyes, PAHs, lindane, DDT, PCP, PCBs	LiP, MnP	Glenn and Gold, 1983; Bumpus et al., 1985; Field et al., 1992; Cerniglia, 1997; Novotný et al., 1997; Beaudette et al., 1998.
Phlebia spp. (WRF)	Polyporales	PAHs, TNT, AmDNT, coal humic acids	Lacc. LiP, MnP	Hofrichter and Fritsche, 1996; Hofrichter and Fritsche, 1997a; Hofrichter and Fritsche, 1997b; Sack et al., 1997; Scheibner et al., 1997a; Scheibner et al., 1997b.
Phlebia sp. b19[b] (WRF)	Polyporales	PCDD/Fs, TNT	Lacc. LiP, MnP	van Aken et al., 1999; Mori and Kondo, 2002a; Mori and Kondo, 2002b; Mori et al., 2003; Kamei et al., 2005; Kamei et al., 2009.
Pleutous ostreatus (WRF)	Agaricales (Pleurotaceae)	PAHs, PCBs, TNT	Lacc., (LiP)[e], (MnP)[e], VP	Bezalel et al., 1996a; Novotný et al., 1997; Scheibner et al., 1997a; Beaudette et al., 1998; Axtell et al., 2000.
Stropharia rugosoannulata (LDF)	Agaricales (Strophariaceae)	PAHs, TNT, synthetic dyes	Lacc., MnP	Scheibner et al., 1997a; Gramss et al., 1999a; Steffen et al., 2000; Steffen et al., 2002a; Baldrian and Šnajdr, 2006.
Trametes versicolor (WRF)	Polyporales	PAHs, PCP, PCBs	Lacc., (LiP)[e], MnP	Field et al., 1992; Logan et al., 1994; Johannes et al., 1996; Novotný et al., 1997; Scheibner et al., 1997a; Beaudette et al., 1998; Majcherczyk et al., 1998; Tuomela et al.,1999.

[a] WRF = white-rot fungus; LDF = litter-decomposing fungus.
[b] Former *Nematoloma frowardii* b19 (Hildén et al., 2008).
[c] International Mycological Assosiation, 2010. Family classification for Polyporales is not as straightforward as for Agaricales.
[d] PAHs = polycyclic aromatic hydrocarbons; TNT = 2,4,6-trinitrotoluene; PCBs = polychlorinated biphenyls; DDT = 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane; PCP = pentachlorophenol; AmDNT = amino-dinitrotoluene; PCDD/Fs = dibenzo-*p*-dioxins and -furans.
[e] Enzyme not detected and/or not directly involved in degradation.

The information gained during the last years permits experts to draw a list of the main facts about fungal degradation of contaminants:

i) Among all fungi, white-rot and litter-decomposing fungi are the most efficient degraders of recalcitrant compounds, an ability attributed to the production of LMEs.

ii) Fungi may also exhibit other enzymatic or non-enzymatic mechanisms involved in the degradation process.

iii) Due to their low substrate specificity, LMEs degrade organic compounds with molecular structures similar to lignin (Fig. 1.7).

iv) Degradation of contaminants occurs during secondary metabolism and, thus, generally under nutrient-starvation conditions (i.e., low levels of nitrogen content; Glenn and Gold, 1983; Reddy, 1995; Pointing, 2001; Gao et al., 2010).

v) The extracellular nature of LMEs enables fungi to degrade molecules larger than the ones degradable by bacteria.

vi) Fungi are able to mineralize organic contaminants or to form low-molecular-mass metabolites which may be co-metabolized by bacteria.

vii) Unlike bacteria, fungi do not assimilate contaminants as a single source of carbon and energy; thus, fungi require an additional carbon source to support their growth, usually a lignocellulosic material.

viii) Fungi tolerate high concentrations of organic contaminants and heavy metals without detrimental effects to their enzyme activity (Baldrian et al., 2000; Baldrian, 2003; Tuomela et al., 2005).

ix) In soil, fungi can cause the humification of organic contaminants, meaning that the compound is bound to humic substances, thereby reducing availability and, thus, toxicity (Bollag, 1992; Bogan et al., 1999).

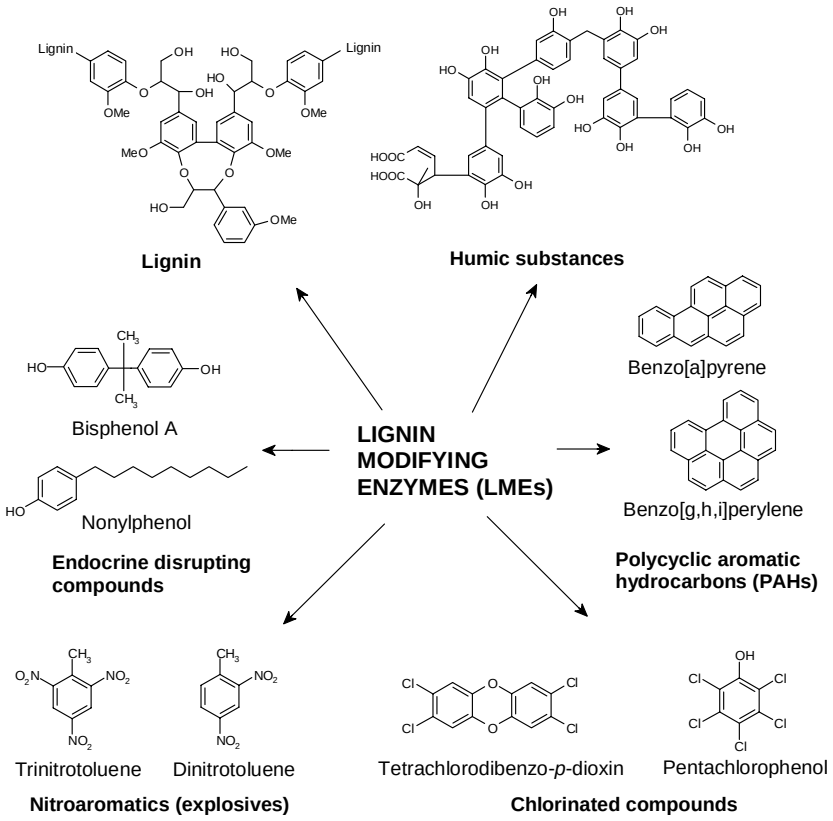


Figure 1.7 Example of various organic compounds which lignin-modifying enzymes of wood-degrading Basidiomycetes are able to attack.

1.5.1 Degradation of PAHs by fungi

The mechanism for LMEs to degrade PAHs is thought to be similar to that of lignin degradation. The breakdown of PAHs yields quinones, free radical intermediates and carboxyl radicals that can undergo further oxidation to form carbon dioxide (Fig. 1.2; Cerniglia and Sutherland, 2001; Singh, 2006). Fungal peroxidases oxidize PAHs with an ionization potential (IP) lower than 8.0 eV in the case of LiP and 7.8 eV in the case of MnP. Unlike peroxidases, laccases are able to oxidize PAHs with an IP lower than 7.55 eV (Singh, 2006; Farnet et al., 2009). However, several authors disagree with the correlation between IP values and the oxidation of PAHs (Majcherczyk et al., 1998; Cañas et al., 2007; Wu et al., 2008). They have proposed that other mechanisms involving the intracellular cytochrome P450 enzyme (Bezalel et al., 1996b) and the MnP-mediated lipid peroxidation play a role in PAH degradation (Kapich et al., 2005; Steffen et al., 2007), especially in the initial attack of the ring. The enzymatic strategy of fungi to degrade PAHs as well as other contaminants depends upon the fungal species and nutrients or the addition of mediators. For instance, the WRF *Irpex lacteus* is able to simultaneously produce MnP and LiP, but only MnP seems to be responsible for PAH degradation, regardless of the nitrogen concentration in the medium (Novotný et al., 2009). In the studies by Johannes et al. (1996) and Majcherczyk et al. (1998), the laccase of *Trametes versicolor* was able to oxidize PAHs independent of the PAH's IP and in the presence of different mediators, such as ABTS and HBT.

1.5.2 Fungal remediation of contaminated soil

To date, numerous studies have addressed the degradation potential of fungal enzymes in liquid media. These studies have elucidated the catabolic pathways of several contaminants and enzymes that are involved (see references in Table 1.3). However, when considering the bioremediation of soils, only a few studies have defined the actual role of enzymes. Unlike in the aqueous phase, the estimation of enzymatic activity in solid matrices is complicated. Currently, the procedure for analysing enzyme activity in soil is based on indirect assays. Enzymes are recovered from the soil using a buffer solution with a pH ranging from 4.5 to 7.0 and the activity is measured by specific protocols (see references in Table 1.4 and the review by Baldrian, 2009). Table 1.4 summarizes the studies addressing the correlation of produced enzymes with soil decontamination. Overall, these studies suggest that, in the complex soil environment, a synergic action between all enzymes - LMEs, intracellular enzymes, and hydrolases – is involved in the biodegradation process. This process is also governed by the type of soil, the concentration of the contaminants, and other environmental factors. The correlation between enzyme activities and degradation is frequently not observed, suggesting the participation of other mechanisms, such as the MnP-mediated lipid peroxidation (Kapich et al., 1999a) or the synergetic action of soil endogenous microbes.

If the conditions are not favourable, for example because of depletion of the substrate, fungi can penetrate and proliferate in the soil to search for lignocellulosic-based resources. In this respect, the formation of mycelial cords, structures which migrate and explore soil cavities and transport adsorbed nutrients, represent an important factor for soil bioremediation (Dix and Webster, 1995; Baldrian, 2008b), although not all the Basidiomycetes produce such cords. Unlike bacteria, the hyphal mode of growth permits fungi to access less available contaminants or even mobilize bacteria to access pollutants in the soil (Wick et al., 2007). In bioremediation, fungal mycelium is directly introduced into some lignocellulosic material (see section 1.4.1).

Table 1.4 Degradation of contaminants in soil by fungi and role of the lignin-modifying enzymes. Modified from Tuomela and Hatakka (2011).

Fungi	Assayed enzymes	Contaminant in soil (mg/kg)	Degradation (%)	Involved enzyme	Substrate (substrate:soil, w:w)	Enzyme extraction	References
3 basidiomycetes isolated from compost	Lacc., LiP, MiP, MnP	Pyrene (100)	56	Lacc.	Straw (1:10)	50 mM acetate buffer, pH 5.0, 4 ºC	Anastasi et al., 2009
Pleurotus ostreatus	Lacc., MnP	Sum of 8 PAHs (80) Addition of 100 mg/kg Cd and Hg	30.2 (with Cd) 68.3 (with Hg)	MnP	Straw (1:2)	50 mM phosphate buffer, pH 7.0, on ice	Baldrian et al., 2000
Trametes versicolor	Lacc.	Atrazine (0.5)	85-98	Slight	Sawdust (1:25)	10 mM phosphate buffer, pH 6.0, 4 ºC	Bastos and Magan, 2009
P. ostreatus	Lacc.	Sum of 16 PAHs (1,900) from creosote	89 (3-ring PAHs) 87 (4-ring PAHs) 48 (5-ring PAHs)	Slight	SMC, MC (1:4)	information not available	Eggen, 1999
Phanerochaete chrysosporium, *P. sordida*, *Trametes* spp.	Lacc., MnP	PCP (> 1,000)	50-65 (*Trametes* spp.; 2-9% formation of PCA) *Phanerochaete* spp.; > 60 % formation of PCA)	Lacc.	Hardwood sawdust + corn grits-rye-corn meal starch (various ratios)	50 mM malonic acid, pH 4.5	Ford et al., 2007a
P. chrysosporium, *T. versicolor*	Total ligninolytic activity, Lacc.	Simazine, trifluralin, dieldrin (10 each)	63-67 (*T. vers*.) 78-79 (*P. chry*.)	Slight	Softwood chips (5:95)	10 mM phosphate buffer, pH 6.5, 40 ºC	Fragoeiro and Magan, 2008
7 WRF and 5 LDF	Lacc., LiP, MnP	3-7 ring PAHs (6.3-53 each)	In average: 14 (by WRF) 26 (by LDF)	Slight	Hardwood cubes + sawdust, straw	Deionized H_20 + 0.1 M K-phosphate-citric acid, pH 4.5 and 7.0	Gramss et al., 1999b
P. ostreatus, *P. chrysosporium*, *T. versicolor*	Lacc., LiP, MnP	Sum of 3 PAHs (150)	95 (*P. ostr*.) 78 (*P. chry*.) 69 (*T.vers*.)	Slight No LiP activity	Straw in tube, separated with nylon web (1:1)	50 mM succinate-lactate buffer, pH 4.5	Novotný et al., 1999
P. ostreatus, *P. chrysosporium*, *T. versicolor*	Lacc., MnP	ANT (50) PYR (50)	ANT-PYR 95-97 (*P. ostr*.) 60-82 (*P. chry*.) 60-53 (*T. vers*.)	MnP (*P. ostr*.) Slight (*P. chry*.)	Polyurethane or pinewood cubed + straw	50 mM succinate lactate buffer, pH 4.5	Novotný et al., 2004
Lentinus edodes	Lacc., MnP	PCP (200)	99 (sterilized soil + fungus) 42 (non-sterilized soil + fungus)	Lacc. and MnP	Sawdust (1:5)	Distilled water, 4 ºC	Okeke et al., 1997
P. chrysosporium	LiP, MnP	PHE (10) PYR (10) BaP (10)	73 (PHE) 51 (PYR) 25 (BaP)	LiP and MnP	Sawdust (1:10)	Distilled water	Wang et al., 2009

WRF = white-rot fungus; LDF = litter-decomposing fungus; Lacc. = laccase; LiP = lignin peroxidase; MiP = manganese independent peroxidase; MnP = manganese peroxidase; SMC = spent mushroom compost; MC = mushroom compost; PCP = pentachlorophenol; PCA = penthachloronoanisole; ANT = anthracene; PYR = pyrene; PHE = phenanthrene; BaP = benzo[a]pyrene.

Fungal bioremediation techniques are commonly suitable for *ex situ* or *on site* applications. To date, field-scale studies have been applied as a solid-phase for the treatment of petroleum hydrocarbons, PAHs, chlorophenols and TNT-contaminated soils (see references in Table 1.5). As in traditional biopiles, fungal-based engineered piles are built to allow irrigation, leachate collection and temperature control. In these field-scale trials, the soil has usually been inoculated with WRF, but in some cases also with LDF (Šašek et al., 2003) or with isolated fungi from the soil endogenous microflora (Li et al., 2002). Regardless of the soil type, in all the trials the target contaminants have been successfully removed, although with some limitations for PAHs (Davis et al., 1993). The substrates used for fungal inoculum are easily biodegradable lignocellulosic materials, often sawdust, woodchips or straw, applied at different soil:substrate ratios. In all cases, the substrate inmobilized fungus has been more prone to efficiently grow into soil than the free mycelium. The lignocellulosic based-inoculum provides constantly nutrients to fungi, which unlike bacteria, do not use the contaminants as source of carbon. In some occasiones even amendments, such as surfactants, have been applied (e.g. Tween 80; Axtell et al., 2000). In general, the pre-grown fungal inoculum is mixed with the contaminated soil or spread on the top of the pile. In this thesis, a novel solid-phase technique is presented in which the fungal inoculum is introduced in a mesh tube. This approach has the advantage of easily re-inoculating the soil with fresh substrate.

Another *ex situ* fungal bioremediation application is the slurry-phase bioreactor, where typically pre-cultivated and homogenized fungal liquid inoculum is added to an aerated and stirred reactor containing 20 - 25% of soil. The nutrients for fungal growth are typically added to the aqueous phase via a synthetically prepared medium (see references in Table 1.5). In some cases, the slurry may be amended with some lignocellulosic substrate, such as dried distiller grain residue from the production of bioethanol (Rubilar et al., 2007). Even if the efficiency of fungal slurry reactors has proven to be higher than that of solid-phase technologies, no field-scale demonstration is yet available. This is likely due to the high costs of the process (e.g., the energy cost). Nevertheless, since slurry fungal reactors have already successfully been applied to degrade PAHs (May et al., 1997; Garon et al., 2004), PCP (Rubilar et al., 2007), and the herbicide hexachlorocyclohexane (Quintero et al., 2007) from soil, and even to remove pharmaceuticals from sewage sludge (Rodríguez-Rodríguez et al., 2010), more studies must be performed in order to improve the technology and make it more economically feasible.

Table 1.5 *Ex situ* applications for fungal bioremediation of contaminated soil.

Field scale biopiles. Modified from Steffen and Tuomela (2010).

Fungus	Contaminant (mg/kg)	Pile size (m; H x L x W)	Soil content per pile (m^3 or t)	Substrate (soil:substrate)	Degradation (%)	Time (days)	References
Trametes versicolor	PCP (900)	0.6x1.35x1.5 (4 piles)	0.5 m^3	Sawdust-cornmeal-starch + wood chips (3:2)	94.4 99.6	518 913	Walter et al., 2005
Agaricus bisporus	PAHs (630)[b]	2.5x1.3x1.35 (1 pile)	0.17 t	Standard mushroom compost (1:4)	68.8	154	Šašek et al., 2003
Cunninghamella sp[a] *Fusarium* sp[a] *Mucor* sp[a] *Phanerochaete chrysosporium*	Petroleum hydrocarbons (49,900)	0.5x8x2 (4 piles)	8 m^3	Chicken excrement-micronutrients-rice husks + wheat bran (6:1)	49 38 (aromatics)	53	Li et al., 2002
Pleurotus ostreatus	TNT (194)	0.3x11x2.4 (1 pile)	4.6 m^3	Rye + cellulose fibre + "Spawn Mate"+ gypsum (2:1). Molasses and Tween 80 as liquid amendments (380 l/pile)	98	62	Axtell et al., 2000
P. chrysosporium	CPs (188)	2x50x30 (4 piles)	1600 m^3	Straw + wood chips + sawdust + pine bark	89	720	Holroyd and Caunt, 1995
P. chrysosporium *P. sordida* *Trametes hirsuta*	PCP (717) PAHs (1,210)[b]	0.25x3x3 (2-7 piles)	2.2 t	Grain-sawdust + aspen wood chips (9:1)	0 - 91 (PAHs)[c] 67 (PCP, *P. chrys.*) 89 (PCP, *P. sord.*) 23 (PCP, *P. chrys* + *T. hirs*)	56	Davis et al., 1993; Lamar et al., 1993; Lamar et al., 1994
P. chrysosporium *P. sordida*	PCP (1-4,434)	0.25x1x1 (2 piles)	0.37 t	Aspen wood chips + peat moss (18:1)	86 (*P. chrys.*) 82 (*P. sord.*)	46	Lamar and Dietrich, 1990

Table 1.5 *Continuation*

Slurry-phase bioreactors

Fungus	Contaminant (mg/kg)	Reactor volume (l)	Soil content (%, w/v)	Substrate	Degradation (%)	Time (days)	References
Bjerkandera adusta	HCH isomers (100, each): α-HCH, β-HCH, δ-HCH and γ-HCH[d]	5	10	Glucose (2 g/l) Peptone (0.4 g/l)	93.4 48.5 43.7 69.1	30	Quintero et al., 2007
B. adusta *Anthracophyllum discolor*	PCP (250)[d]	0.125	10	DDGS	18 (*B. adus.*) 33 (*A. disc.*)	14	Rubilar et al., 2007
P. chrysosporium	16 PAHs (41,196 total)	3.5	2.5	Nitrogen-limited medium with 0.1 % Tween 80	44.7	36	May et al., 1997
Absidia cylindrospora	FLU (100)[d]	0.100	20	Nutrient GS medium	54	12	Garon et al., 2004
T. versicolor	NAP and CBZ (670 each)[e]	0.120	25	Nutrients from sewage sludge	47 (NAP) 57 (CBZ)	1	Rodríguez-Rodríguez et al., 2010

[a] Indigenous fungus enriched.
[b] Sum of 16 PAH compounds.
[c] Reduction of various PAH compounds: the more rings, the less degradation.
[d] Artificially spiked soil.
[e] Artificially spiked sewage sludge.
HCH = hexachlorocyclohexane; PCP = pentachlorophenol; PAHs = polycyclic aromatic hydrocarbons; FLU = fluorene; GS = Golzy and Slonimski medium (Garon et al., 2004); NAP = naproxen; CBZ = carbamazepine; DDGS = dried distiller grains; TNT = 2,4,6-trinitrotoluene; CPs = chlorophenols.

2 BACKGROUND AND AIMS OF THE STUDY

The starting point of this study was the development and demonstration of two fungal bioremediation *ex situ* applications to treat contaminated soil under different conditions.

The first case took place in Spain, when the single-hulled oil tanker Prestige sank on the Cap Finisterre (A Coruña, NW Spain) on 19 November 2002 and spilt 64,000 tons of heavy fuel oil (N° 2, M100) into to the sea. Eventually, the oil reached the shorelines, estuaries and marshes, affecting 1,900 km of the coast (CEDRE, 2006). After investigations, researchers suggested that traditional *in situ* bioremediation techniques were likely not suitable for recovering the shorelines due to the high content (around 50%) of the aromatic fraction of the Prestige fuel oil (Fernández-Álvarez et al., 2007; Gallego et al., 2007). Moreover, the large amount of wastes generated after the first emergency clean up using high pressure hot water flushing indicated that the best biological technologies for treating the soil would be those performed *ex situ* or *on site*. In this respect, a project was initiated at the Chemical Engineering Department of the University of Santiago de Compostela (Spain) to develop a novel system for treating the contaminated marine and shoreline areas based on the use of white-rot fungi (WRF) in a slurry-phase bioreactor, which was focused on the most recalcitrant and toxic compounds derived from the Prestige oil: the polycyclic aromatic hydrocarbons (PAHs).

Considering the fact that agitation and saline conditions may not be the most suitable environment for fungi to grow, the specific aims of the first part of this thesis were as follows:

- to evaluate the effects of saline conditions on the growth and ligninolytic activity of WRF.
- to study the fungal degradation of PAHs in a stirred slurry bioreactor under saline and non-saline conditions in both small (100 ml) and large (5 l) laboratory scale reactors.
- to determine the most suitable parameters for performing a slurry-phase bioreactor, focusing on the type of fungal inoculum, nutrient amendments and biomass concentration.

The starting point for the second part of the thesis arose from the environmental problems in former wood preservation sites and sawmill areas in Finland. Approximately 550 sites are potentially contaminated with dibenzo-*p*-dioxins and dibenzofurans, but also with other harmful substances, such as chlorophenols. Consequently, around 100 of these sites require urgent treatment to prevent health risks or damages to the environment (Haavisto, T. Finnish Environment Institute, personal communication). Nowadays, the only applicable treatment for dioxin-contaminated soil is excavation, followed by thermal

treatment at very high temperatures. However, the large content of organic matter derived from wood debris hinders the efficiency of the whole thermal process (Rantsi, R. Niska ja Nyyssönen Oy, personal communication). In light of this situation, a project focused on the degradation of organic matter was initiated. The only organisms capable of decomposing all of the wood components are wood-degrading basidiomycetes; thus we planned to pre-treat dioxin-contaminated soil with a fungal cultivation, aiming at the reduction of organic matter content in order to aid the efficiency of the subsequent thermal treatment. The growth of introduced fungi in soil is very challenging due to the intricate relations with other soil microbes and soil properties; thus, the pre-selection of fungi was done carefully. We first considered litter-decomposing fungi, whose natural habitat is soil and which posses the necessary arsenal of enzymes to degrade lignocellulose from litter. Despite their assumed limitation at colonizing soil, white-rot fungi (WRF) and brown-rot fungi (BRF) were also included, due to their extensive degradation of wood components and the contamination tolerance of WRF. In addition, we included fungi whose habitats overlap with those of LDF and WRF. Mycorrhizal fungi were excluded from our study because of their lack of extracellular ligninolytic enzymes.

At the same time, the bark from Scots pine (*Pinus sylvestris*) was selected as the lignocellulosic material to introduce fungi into contaminated soil based on the positive results achieved in a previous study (Steffen et al., 2007). The lack of more specific knowledge about the use of pine bark as fungal inoculum led us to examine closely this lignocellulosic material and to develop a suitable carrier to introduce fungal-bark inocula into the soil, while preventing the addition of organic matter into the soil. Lastly, considering the absence of attention to fungal bioremediation at large scale, the aims were also to scale up the process. More specifically, the aims of the second part of this thesis were as follows:

- to select basidiomycetes that compete and grow in non-autoclaved contaminated soil.
- to study the fungal ligninolytic and hydrolytic enzymes in contaminated soil and pine bark.
- to degrade soil organic carbon from several contaminated sawmill soils.
- to assess the degradability of Scots pine bark and its application as a lignocellulosic substrate for fungal bioremediation.
- to develop and scale up a fungal technology to pretreat soil containing high organic matter.

Finally, based on the results of the two methods, conclusions on the applicability of fungal remediation techniques and suggestions for further studies and development were summarized.

3 MATERIALS AND METHODS

3.1 Schematic overview of the thesis

Three constitutive set of experiments were set up in this study (Fig. 3.1): i) degradation of PAHs in a soil slurry-phase bioreactor (I and II), ii) solid-phase pretreatment of contaminated soil (III and IV), and iii) chemical characterization of Scots pine (*Pinus sylvestris*) bark during fungal degradation (V).

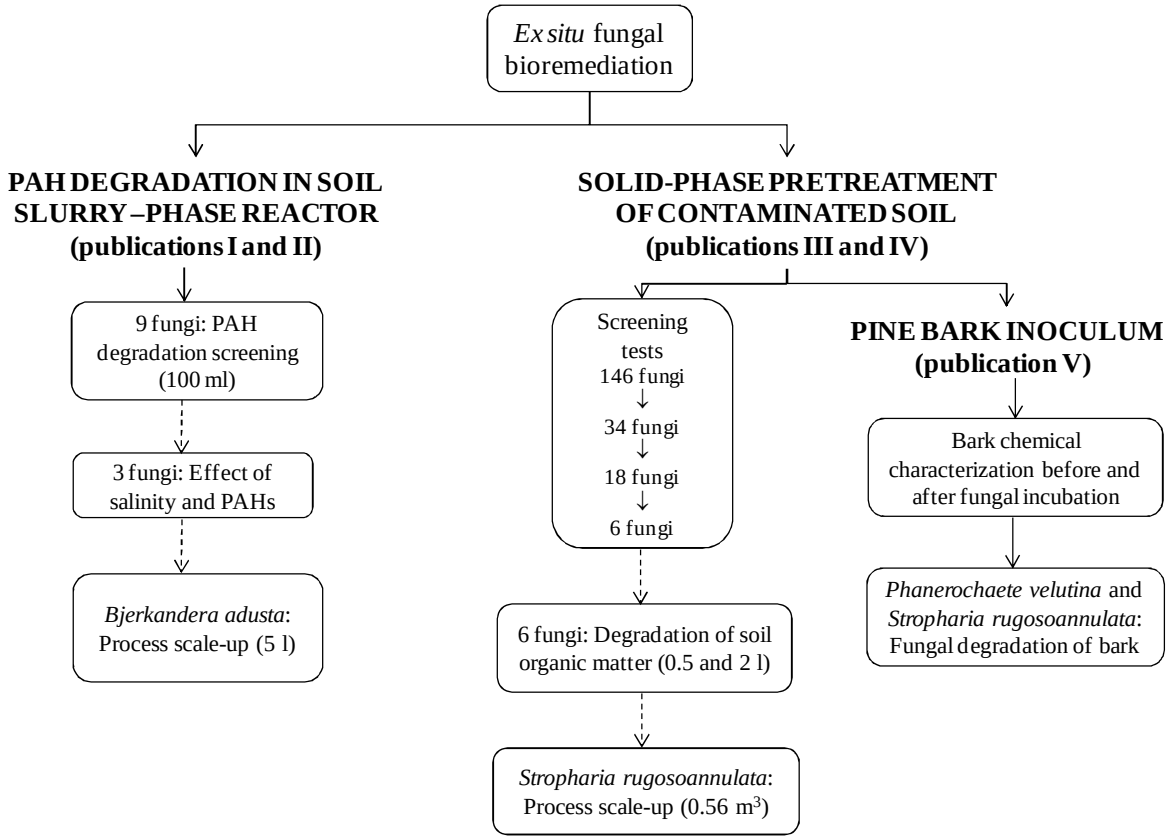


Figure 3.1 Schematic overview of the experiments.

3.2 Screening tests and fungal strains

Initial slurry-phase experiments included nine white-rot fungi, which were obtained from the culture collection of the Chemical Engineering Department of the University of Santiago de Compostela (Spain) to evaluate the fungal potential to degrade PAHs under saline and non-saline conditions (Table 3.1). The screening tests provided three strains of fungi for assessing salinity and the PAH concentration affecting fungal growth and ligninolytic activity (Table 3.1). *Bjerkandera adusta* BOS55 ATTC 90940 was selected for the final scale-up of the slurry-phase reactor.

The preliminary screening for the solid-phase pretreatment of contaminated soil included 146 fungi capable of growing in non-sterile low organic matter contaminated

sawmill soil (LOM, sawmill A; Annexed table shows the full list of fungi). The fungi were obtained from the Fungal Biotechnology Culture Collection (FBCC) at the University of Helsinki's Department of Food and Environmental Sciences. Among them, 55 were white-rot fungi (WRF), 12 brown-rot fungi (BRF), and 52 litter-decomposing fungi (LDF). The residual 10 belonged to a group whose habitat overlaps between WRF and LDF (WRF-LDF), and the habitat of 17 strains was unknown. The second screening, which included 34 strains from the previous 146 screened fungi, considered the growth of fungi in high organic matter contaminated sawmill soil (HOM, sawmill soil B, Table 3.2). Thereafter, a screening assay with 18 fungi evaluated the ligninolytic capability of fungi in indicator agar plates. The last screening assay was based on the production of laccase, manganese peroxidase and polysaccharide-degrading enzymes (endo-1,4-β-glucanase, endo-1,4-β-xylanase and endo-1,4-β-mannanase) in Scots pine bark. Six fungi were further studied for their potential to degrade organic matter in various soils (Table 3.1). Finally, *Stropharia rugosoannulata* FBCC475 11372 B was selected to study the scale-up of the treatment process.

3.3 Contaminated soils

In total, eight soils were used in this study (Table 3.2). After collection, soils were air-dried, sieved (< 2mm) and stored at 4 °C.

For the slurry experiments, non-contaminated forest and saline marsh soils were used. The marsh soil came from Ría de Arosa, located in the Lower Rías of the Galician coast (Northwest Spain), and forest soil at a depth of 20 cm from a grassy area in an oak grove in Santiago de Compostela (Galicia). Both soils were spiked with a stock solution of four PAH compounds to reach a total concentration of 200 mg/kg (Table 3.2).

Four contaminated soil batches from a former sawmill, soil from a shooting range and soil from a landfill site, all in Finland, provided the soil samples for the second part of this study. The sawmill soils came from a site where timber had been treated with the preservative Ky-5 by dipping it into pools dug into the soil. The sawmill soils have been contaminated with different levels of polychlorinated dibenzo-*p*-dioxins and -furans [PCDD/Fs; 0.06 - 2.1 mg International Toxic Equivalent (I-TEQ) per kg of soil]. The organic matter content and PCDD/F concentration varied between the soil batches and, accordingly, they were designated as A, B, C and D (Table 3.2). The soil from a shooting range in Helsinki (Finland) was contaminated with lead (Pb; 700 – 1,200 mg/kg) and PAHs (sum of 16 PAHs; 150 mg/kg). The soil obtained from a landfill in Jyväskylä (Central Finland) was contaminated with petroleum hydrocarbons (Table 3.2). Carbon, nitrogen, pH and organic matter were determined for all of the soils. Additionally, the cation exchange capacity (CEC) and various anions of the forest and marsh soil were analyzed (Table 1 in I).

Table 3.1 Fungi studied in detail in slurry- and solid-phase experiments

Fungus	Ecophysiological group[a]	Experiment	Soil[c]
Slurry phase bioreactor			
Phanerochaete chrysosporium BKM-F-1767	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Phanerochaete sordida YK-624	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Polyporus ciliatus ONO94-1	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Stereum hirsutum PW93-4	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Lentinus tigrinus PW94-2	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Bjerkandera adusta BOS55 ATTC 90940	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I) and technology scale-up (II)	Forest and marsh
Irpex lacteus Fr. 238 617/93	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Pleurotus eryngii CBS 613.91	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Phlebia radiata WIJSTER94-6	WRF	Salinity and PAHs tolerance (I)[b] PAH degradation (I)	Forest and marsh
Solid-phase pretreatment of contaminated soil and bark degradation			
Agrocybe praecox FBCC 476 TM 70.84	LDF	Soil organic carbon degradation (III)	Sawmill A
Gymnopilus luteofolius FBCC466 X9*	LDF	Soil organic carbon degradation (III and IV) Enzyme activities in soil (III)	Sawmill A, C
Hypholoma fasciculare FBCC1034 CCBAS 287	WRF-LDF	Soil organic carbon degradation (III)	Sawmill A
Phanerochaete velutina FBCC941 T244i	WRF	Soil organic carbon degradation (III and IV) Enzyme activities in soil (III) Bark degradation (V)[b]	Sawmill A, B, C
Stropharia rugosoannulata FBCC475 11372 B	LDF	Soil organic carbon degradation (III and IV) Technology scale-up (IV) Enzyme activities in soil (III) Bark degradation (V)[b]	Sawmill A, B, C Landfill
Sphaerobolus stellatus FBCC253 PO203	WRF-LDF	Soil organic carbon degradation (III and IV)	Sawmill A, D Shooting range

* Former *Pholiota* sp. (Hofrichter and Fritsche, 1996).

[a] WRF = white-rot fungi; LDF = litter-decomposing fungi; WRF-LDF = fungi whose habitat overlaps with those of WRF and LDF.

[b] No soil used.

[c] Soil properties and contamination are shown in Table 3.2

Table 3.2 Origin, properties and contamination of soils used in this study.

Soil origin	pH	Carbon %	Nitrogen %	Organic matter[a] %	Contaminant (mg/kg)	Publication
Forest	4.04	7.85	0.55	9.8	Mix of 4 PAHs (200)[b]	I
Marsh	5.57	2.12	0.55	1.6 - 2.2	Mix of 4 PAHs (200)[b,c]	I, II
Sawmill A	4.6	4	0.08	9	PCDD/Fs (2.1 I-TEQ)[d]	III
Sawmill B	4.3	48	0.41	84	PCDD/Fs (2.1 I-TEQ)[d]	III
Sawmill C	4.3	46	0.41	82	PCDD/Fs (0.06-0.07)[d]	IV
Sawmill D	4.3	42	0.43	82	PCDD/Fs (0.06-0.07)[d]	IV
Shooting range	3.9	16	0.72	28	Pb (700-1,200)[d] 16 PAHs (150)[d]	IV
Landfill	7.1	8.3	0.13	14	Petroleum hydrocarbons C_{10}-C_{40}: 10,000-34,000)[d]	IV

[a] Organic matter was determined by loss on ignition at 440 °C over 5 hours.
[b] Artificially contaminated soil with PAHs: phenanthrene, fluoranthene, pyrene, chrysene (I).
[c] For the 5 l slurry reactor marsh soil was artificially contaminated with dibenzothiophene, fluoranthene, pyrene, chrysene (II).
[d] Analyses of contaminants were not done by the author of this thesis.

3.4 Configuration of slurry- and solid-phase reactors

3.4.1 Slurry-phase reactors (I and II)

Initially, the degradation of four PAHs (phenanthrene, fluoranthene, pyrene and chrysene) by nine fungi was studied in a 100 ml slurry reactor with 10% soil (w/v) cultivated during 30 days. The reactors were filled with 20 ml of autoclaved soil slurry and operated at 120 rpm for gentle agitation (Fig. 3.2 a). The evaluation of PAH degradation took place under non-saline (using forest soil and culture medium; see detailed description of the medium in publication I) and saline conditions (using marsh soil and marsh water; see marsh water composition in publication I). The fungal inoculum was added to the soil slurry as free mycelial suspension. PAH losses due to volatilization were estimated with six replicates of abiotic controls under the same conditions described above. PAH degradation by fungi were calculated considering the residual PAHs in the abiotic controls on day 30.

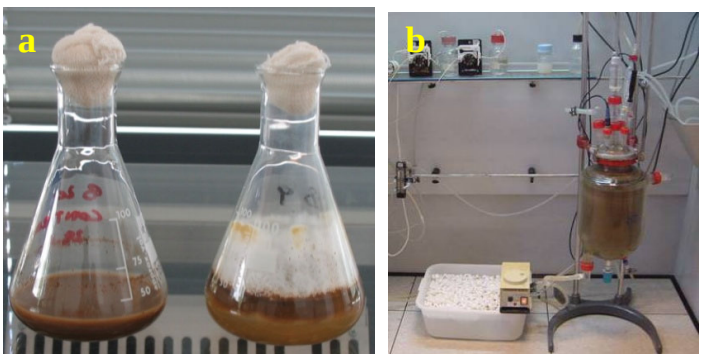


Figure 3.2 Reactors used for fungal degradation of PAHs in slurry-phase. a) 100 ml flask filled with 20 ml of soil slurry (left, abiotic control; right, 9-day-old culture of *Lentinus tigrinus;* I). b) 5 l Alamo reactor used to scale-up the process (II).

The optimization and scale-up of the slurry technology was carried out in a 2 l fermentor (1.5 l working volume, BIOSTAT-MD, B. Braun-Biotech, Germany) and in a 5 l stirred tank reactor (4 l working volume, Hermanos-Alamo S.L., Spain; Fig. 1.3 and 3.2.b) with *Bjerkandera adusta*. In the Alamo reactor, soil slurry was mixed with a turbine propeller at 250 rpm. The temperature and pH were maintained at 30 ºC and 4.5 - 5.5, respectively. The cooling system (5 °C) was installed for the gas outlet to minimize losses of volatile organic compounds. Air was blown into the reactor at 4 l/min. This reactor was used to study the influence of several parameters, such as the type of fungal inoculum, fungal biomass and glucose addition, on the treatment of PAH-contaminated marsh soil. The soil was artificially contaminated with dibenzothiophene, fluoranthene, pyrene and chrysene and treated as slurry of 10% solid fraction (w/v). PAHs were extracted with a hexane:acetone mixture (1:1; v/v) added to a soil slurry sample, of different volume depending on the experiment, and vigorously mixed. Extracted PAHs were analysed by high performance liquid chromatography (HPLC). Analytical and chromatographic conditions are described in publication I and II. To study the influence of agitation on *B. adusta*, various operational parameters - pH, oxygen partial pressure, glucose consumption, redox potential and MnP activity - were monitored during the soil slurry treatment in the Biostat fermentor in the absence of contaminants.

3.4.2 Solid-phase pretreatment (III and IV)

The solid-phase pretreatment of contaminated soil was performed in reactors with different sizes: Small (0.5 l), medium (2 l) and large (560 l; Fig. 3.3). The experimental conditions are summarized in Table 3.3. In all of the experiments, pine bark, previously autoclaved for 20 min at 121 ºC, was inoculated with homogenized liquid mycelium and introduced into the soil. For the small- and medium-scale experiments, fungal bark inoculum was introduced either at the bottom or in the middle of the soil layer, respectively. The soil was continuously aerated with moist air driven through a tube positioned inside the soil layer. Exhaust CO_2 was trapped in 2 M NaOH in the small-scale experiment, and measured on line by mass spectrometer in the medium-scale experiment.

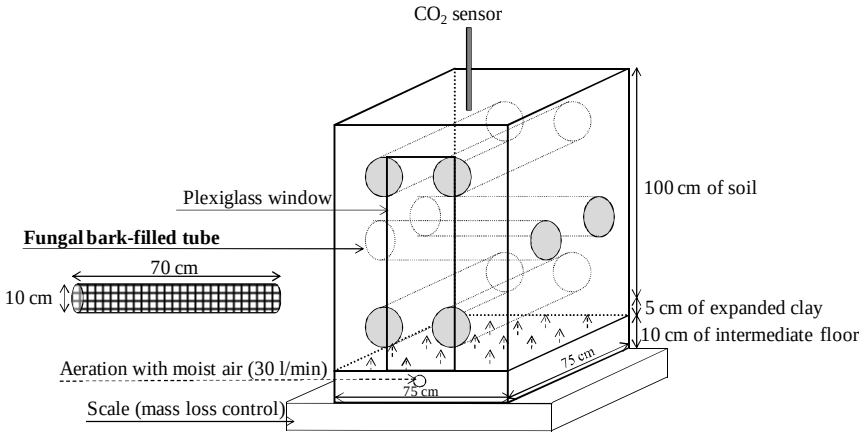


Figure 3.3 Bioreactor configuration at large scale for pretreatment of contaminated sawmill soil D with *Stropharia rugosoannulata* (IV). Total volume of the reactor was 0.56 m^3.

A reactor (0.56 m^3) made of particleboard was specially built for this study (Fig. 3.3). The reactor with an open top and perforated floor was filled with 300 kg of contaminated soil (sawmill soil D, Table 3.2) and placed it on the top of a scale to control mass loss due to the fungal degradation of organic matter. The production of CO_2 was continuously recorded using a CO_2 sensor placed above the soil surface. The moisture content of the soil was kept constant by passing air (30 l/min) through a water tank before it entered into the soil. Water content at the beginning and at the end of the six-month treatment was 71%. To prevent channelling and distribute the air evenly, a 5 cm layer of expanded clay was placed on the bottom of the reactor. Six plastic mesh tubes (from now on "fungal tube") were used to introduce the fungal bark inoculum into the soil (1.5 kg in each tube; Fig. 3.3). The fungal tube prevented an increase of organic material into the soil and permitted the direct contact of mycelium with the soil.

Table 3.3 Experimental conditions for solid-phase pretreatment of contaminated soil.

Parameters	**Small scale (I)**	**Medium scale (II)**	**Large scale (II)**
Studied fungal strains	6	4	1
Soil	Sawmill A and B	Sawmill C and D Shooting range Landfill	Sawmill D
Reactor size (l)	0.5	2	560
Amount of soil (wet matter)	70 - 72 g	700 g	300 kg
bark inocula:soil (w/w)	21:100	14:100	3:100
Placement of bark in soil	bottom	middle	tubes
Aeration flow (l/min)	not monitored	70	30
Moisture control	Moist air and sealed bottles	Moist air and sealed bottles	Moist air and tarpaulin sheet
Respiration activity	CO_2 trapping and titration	Mass spectrometer	CO_2 sensor above soil
Duration (days)	84 - 96	53 - 90	180

3.5 Bark characterization and degradation (V)

The lignocellulosic material used as an inoculum carrier and substrate for fungi in the solid-phase experiments was the bark from Scots pine (*Pinus sylvestris*). Bark was characterized before and after the incubation with *Phanerochaete velutina* or *Stropharia rugosoannulata* for 15, 30, 45, 60, 75 and 90 days. The fungi were incubated in 2 l glass bioreactors (Fig. 3.4). Bark was subjected to various preparations and extractions, according to the analysis performed. For the analysis of lignin, cellulose, hemicellulose, carbohydrates, and extractives bark was dried and ground in a laboratory mill. The cellulose and hemicellulose were determined after neutral and acid detergent extraction (van Soest, 1963) and lignin according to the TAPPI standard method T222 om-88 (1998). Acid methanolyses extraction was used to determine carbohydrates. Bark extractives were extracted with an acetone:water mixture (95:5; v/v) using an Accelerated Solvent Extraction apparatus (ASE; Dionex Corp., USA). Extractives and carbohydrate samples

were silylated prior to analysis with gas chromatography - mass spectrometry (GC-MS). Analytical and chromatographic conditions are described in publication V.

For the analysis of enzyme activities, bark was extracted with a 25 mM sodium phosphate buffer (pH 7.0). MnP analysis was based on the formation of Mn^{3+} malonate complexes at 270 nm and laccase on the oxidation of ABTS at 420 nm (Wariishi et al., 1992; Eggert et al., 1997). The activities of the hydrolytic enzymes endo-1.4-β-glucanase, endo-1.4-β-xylanase, and endo-1.4-β-mannanase were determined with Remazol Brilliant Blue R (RBBR) dye-coupled substrates of carboxymethylcellulose, birch wood xylan, and carob-galactomannan, respectively, according to Baldrian et al. (2005) and the substrate supplier's protocol (Megazyme, Ireland). A detailed description of the enzyme extractions and assays is presented in publications III and V.

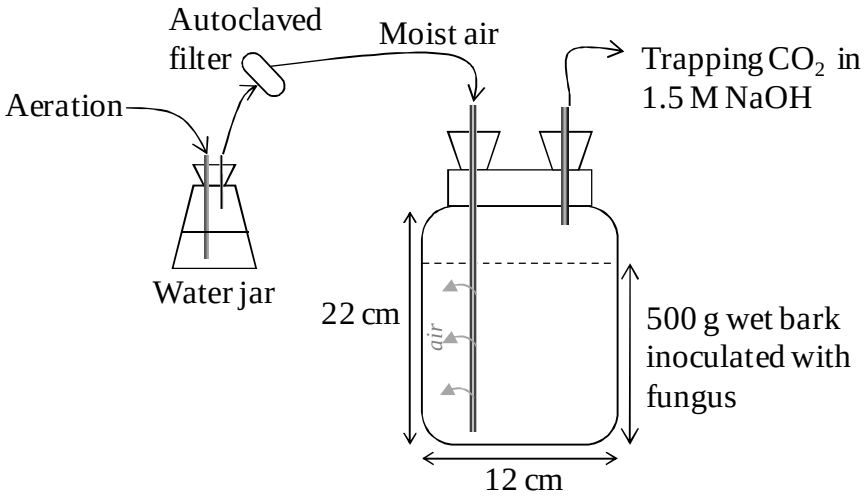


Figure 3.4 Configuration of the 2 l bioreactor used for fungal degradation of Scots pine bark with *Phanerochaete velutina* and *Stropharia rugosoannulata* (V).

3.6 Inocula preparation and analytical methods

The procedures for preparing liquid and solid inocula and for determining soil properties, as well as all the analytical methods used in this study, are summarized in Table 3.4 and described in more detail in the original publications I-V.

Table 3.4 Methods used in this study.

Method	Described in
Plate screening tests of fungal strains	
· visual monitorization of fungal growth in contaminated soil	III
· indirect enzyme activity in agar with RBBR, ABTS, HA, MnCl and CMC	III
Cultivation of fungi in	
· liquid cultures	I, II, III, IV and V
· slurry-phase cultures	I and II
· solid-phase bark cultures	III, IV and V
· solid-phase soil cultures	III, IV and V
Soil preparation	I, II, III and IV
Characterization of soil	
· pH, organic matter, dry matter, carbon, nitrogen	I, II, II and IV,
· cationic exchange capacity and ions	I and II
Extraction of PAHs	I and II
Analysis of PAHs by high performance liquid chromatography (HPLC)	I and II
Respiration activity of fungi and microorganisms in soil and bark	
· CO_2 trapped in NaOH and analysed by titration with HCl	III and V
· online CO_2 analysed by mass spectrometry	IV
· continuous monitoring of CO_2 with a CO_2 sensor	IV
Soil organic carbon loss calculations	III and IV
Examination of salinity and PAHs effect by decolourization of Poly R-478	I
Mycelia observation with microscope	I
Extraction of enzymes	
· from slurry-phase after centrifugation	II
· from soil and bark using 25 mM sodium phosphate buffer (pH 7.0)	III, IV and V
Enzymes assays	
· indirect measurement of oxidative activity with Poly R-478 and wood chips agar plate	II
· spectrophotometric measurement of lignin-modifying enzymes	II, III, IV and V
· cellulase and hemicellulase activities with RBBR-coupled substrates	III, IV and V
Chemical determination of bark constituents	
· cellulose and hemicellulose by neutral and acid detergent extraction	V
· Klason and acid-soluble lignin	V
· non-cellulosic carbohydrates by acid methanolysis	V
· 95% acetone:water (v/v) extraction of extractives using an Accelerated Solvent Extraction apparatus (ASE)	V
· analysis of carbohydrates and acetone extractives by gas chromatography-mass spectrometry (GC-MS)	V
Statistical analyses with R (ANCOVA analysis) and SPSS (ANOVA analysis) programs	I and III

4 RESULTS AND DISCUSSION

4.1 Soil slurry-phase degradation of PAHs (I and II)

4.1.1 Selection of fungi: tolerance of salinity and PAHs (I)

Saline conditions were prepared with 10% (w/v) marsh soil slurry (cation exchange capacity 115.53 $cmol_c/kg$) and marsh water (10‰ salinity). These results were compared with non-saline conditions prepared with 10% forest soil slurry (cation exchange capacity 4.98 $cmol_c/kg$). Both the forest and marsh soils were spiked with a PAH solution containing phenanthrene, fluoranthene, pyrene and chrysene to achieve a final concentration of 50 mg/kg for each PAH. PAH losses occured during 30-day incubation of the abiotic controls, primarly by volatilization. The loss in PAH concentration in forest soil slurry was 32%, 23%, 25%, and 7% and in marsh soil slurry was 45%, 22%, 0%, 7% for phenanthrene, fluoranthene, pyrene and chrysene, respectively. PAH degradation by fungi was considered as the dissapearance of the original PAH concentration and it was calculated considering the abiotic losses.

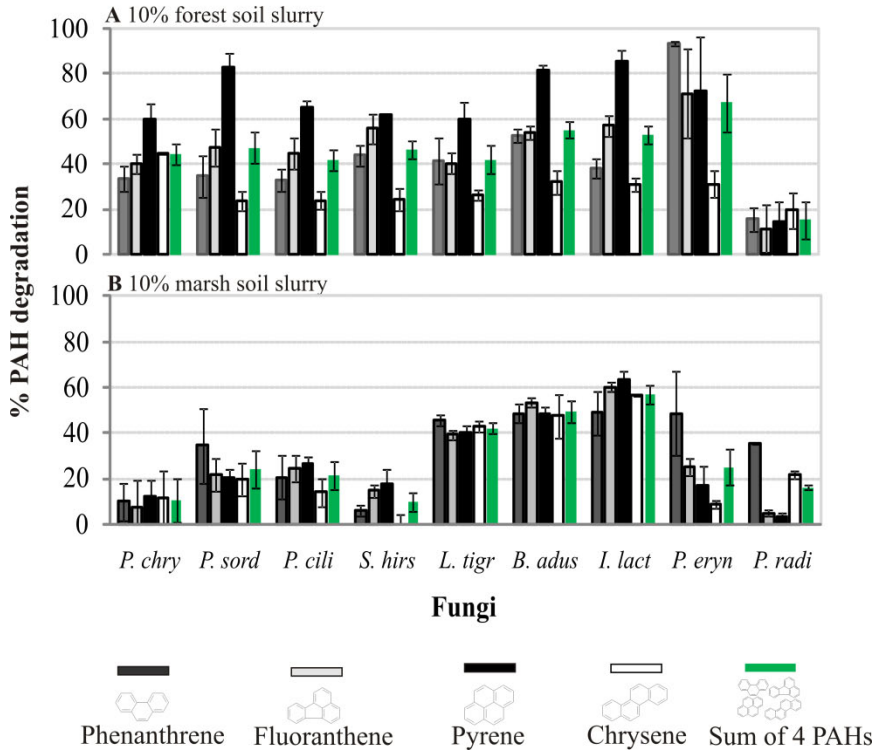


Figure 4.1 Degradation of phenanthrene, fluoranthene, pyrene and chrysene by various white-rot fungi in soil slurry- phase under non-saline (A) and saline (B) conditions during 30 days of incubation. The error bars represent standard deviations of four replicates.

In general, fungi had a lower PAH degradation ability in marsh soil than in forest soil, suggesting some intolerance to saline conditions (Fig. 4.1 and Tables 4 and 5 in I). The strongest inhibition occurred for *Pleurotus eryngii*, which degraded 71% of fluoranthene

and 73% of pyrene in forest soil, while in marsh soil the levels of degradation were only 25% and 17%, respectively (Fig. 4.1). In contrast, salinity did not affect *Irpex lacteus*, *Bjerkandera adusta* and *Lentinus tigrinus*, which, on average, were able to degrade the four PAHs by 57%, 49% and 42%, respectively. Such levels of degradation were the same as that achieved in forest soil: 53%, 55% and 42%, respectively. The other fungi were influenced mostly by saline conditions in which a negligible degradation was detected in marsh soil slurries (Fig. 4.1). *Phlebia radiata*, which produces all of the important LMEs (MnP, LiP and laccase) necessary to degrade wood (Hatakka, 1994; Hofrichter et al., 2001) and many organic contaminants (e.g., TNT; van Aken et al., 1999), degraded only 16% and 15% of PAHs in marsh and forest soils, respectively. Thus, the soil itself or the slurry conditions inhibited the ligninolytic capacity of *P. radiata*.

To date, only anamorphic fungi (Wang et al., 2008) or fungal consortia isolated from soil (Li et al., 2008) have been applied for the bioremediation of PAHs in soil slurry. Regardless of slurry conditions, *B. adusta* and *I. lacteus* have been extensively studied in both liquid and soil, attaining a similar level of degradation as in this study (Novotný et al., 2009). Novotný et al. (2000) have obtained lower levels of degradation for fluoranthene and pyrene (25% and 52%, respectively) in a soil microcosm with *I. lacteus* than those obtained in this study (57 - 60% for fluoranthene and 63 - 86% for pyrene). Likewise, the degradation by *B. adusta* in a slurry phase (50 - 55% average of the sum of 4 PAHs) is in accordance with other work using this fungus to treat various PAHs from actual contaminated soil in a solid-state, attaining approximately 70% degradation (Grotenhuis et al., 1998). In comparison with the study by Gramss et al. (1999b), which evaluates the fungal degradation of five PAHs (phenanthrene, anthracene, fluoranthene, pyrene and perylene) in soil, *B. adusta* achieved a ten-fold higher level of degradation in this study.

Unlike the other fungi, the degradation performance of *L. tigrinus* in PAH-contaminated soil has been rarely addressed (Covino et al., 2010a). As occurred to Covino et al. (2010a) the least degraded PAH was chrysene. This confirms the recalcitrance nature of this PAH due to the lower water solubility (0.0006 mg/l) and relatively higher $\log K_{oc}$ (5.49). Furthermore, Covino et al. (2010b) has proven that the PAH degradation ability of this fungus in liquid cultures is enhanced by agitation. Additionally, it has been suggested that MnP of *L. tigrinus* may be the predominant enzyme participating in PAH degradation, although the cytochrome P450 monooxygenase and the epoxide hydrolase may also be involved in the degradation process (Bezalel et al., 1996b; Bezalel et al., 1997; Covino et al., 2010b).

In accordance with the screening results, the enzymatic system of *I. lacteus*, *B. adusta* and *L. tigrinus*, responsible for PAH degradation, was not affected by saline conditions. To corroborate such halotolerance, a study was carried out to assess the ligninolytic activity following the decolourization of Poly R-478 dye added to a liquid medium (no soil was used). Decolourization of Poly R-478 serves as an indirect measurement of the ligninolytic activity of each fungus. The medium was prepared with various proportions (100%, 50%,

25% and 0%; v/v) of sea water which had three-fold higher salinity (32‰) than marsh water (10‰). The decolourization efficiency was followed as the change in the absorbance ratio at 520 nm and 350 nm (A_{520}/A_{350}). The statistical analyses of covariance confirmed that the decolourization rate varied with the sea water content (ANCOVA, interaction between time and salinity, $F_{1,538}$ = 3.87, P<0.05) and between the fungal strains (ANCOVA, interaction between time and strains, F3.538 = 99.77, P<0.001). The response of different fungi to salinity was also variable (ANCOVA, three-way interaction between time, salinity and strains, F3,538 = 24.31, P<0.001). The fungi least affected by 100% sea water were *L. tigrinus* and *I. lacteus*, whereas *B. adusta* was completely inhibited (Fig. 4.2). At 75%, *B. adusta* was no longer affected, but the decolourization started after an initial lag time of approximately 12 days, similarly as observed in the control of 0% sea water (Fig 4.2). No inhibition of either growth or ligninolytic potential was found at sea water levels ≤ 75%. These results showed that some WRF are halotolerant, while others may be affected by hyper-saline conditions. Terrestrial basidiomycetes are poorly represented in saline environments where mostly anamorphic ascomycetes are prevalent, especially from the genus *Penicillium* (Hujslová et al., 2010). Nevertheless, it has been demonstrated that various terrestrial basidiomycetes withstand a salt concentration below 10% (Tresner and Hayes, 1971; Li et al., 2002). Unlike basidiomycetes, ascomycetes are less efficient in bioremediation applications, thus, in this study, it was important to find white-rot fungal species with a high salt-tolerance in order to apply the bioremediation of marine environments.

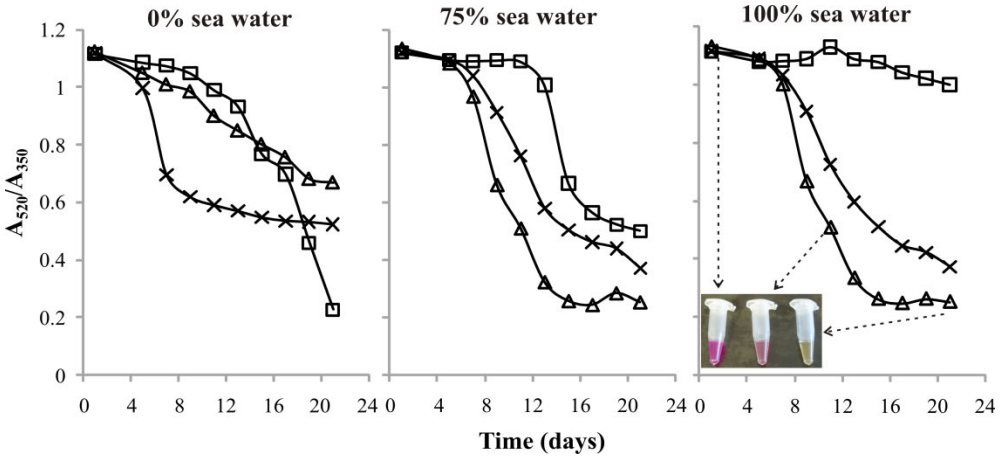


Figure 4.2 Poly R-478 decolourization ratio (A_{520}/A_{350}) used as an indirect measurement of ligninolytic activity of the WRF *Lentinus tigrinus* (✕), *Bjerkandera adusta* (□) and *Irpex lacteus* (△) incubated in liquid media using 0%, 75% and 100% (v/v) of sea water.

Only a few studies have evaluated the ligninolytic capacity of WRF in saline conditions. These studies revealed that several white-rot species with a hypersaline tolerance (e.g., *Phlebia* sp.) are not only able to efficiently decolorize Poly R-478 but also to modify lignin under saline conditions (Li et al., 2002; Li et al., 2003). Furthermore, the production of MnP by *Phlebia* sp. MG-60, isolated from a mangrove, correlated with the decolourization of Poly R-478 and the reaction was enhanced by sea salt addition.

The PAH tolerance of *L. tigrinus*, *I. lacteus* and *B. adusta* was also assessed during the decolourization of Poly R-478, which was added to a medium with various PAH concentrations. For individual compounds (dibenzothiophene, fluoranthene, pyrene and chrysene), the concentrations were 0, 1, 5, 10 and 50 mg/l. These concentrations would correspond to 0, 10, 50, 100 and 500 mg/kg in a slurry phase performed with 10% soil (w/v). None of the fungi tolerated the highest PAH concentration of 50 mg/l (Fig. 2 in I). This kind of contamination corresponds to an extremely contaminated site with 500 mg/kg of each PAH. Commonly, such a high contamination is not found in actual contaminated sites, where, in general, the concentration of a single PAH compound is around 100 mg/kg (Morillo et al., 2007; Kaszubkiewicz et al., 2010), corresponding to 10 mg/l in liquid cultivation. At this concentration the ligninolytic system of all three fungi was not inhibited, as it was indicated by the progressively decrease of the absorbance of Poly R-478 at 520 nm (Fig 4.3). A lower PAH concentration did not necessarily enhance the decolourization rate, but the lag phase was shortened (Fig. 2 in I). Unlike the effect of heavy metals on the physiology of WRF (Baldrian, 2003), little is known about the mechanism operating in some WRF species when they tolerate a high concentration of PAHs (Richter et al., 2003; Kim et al., 2010).

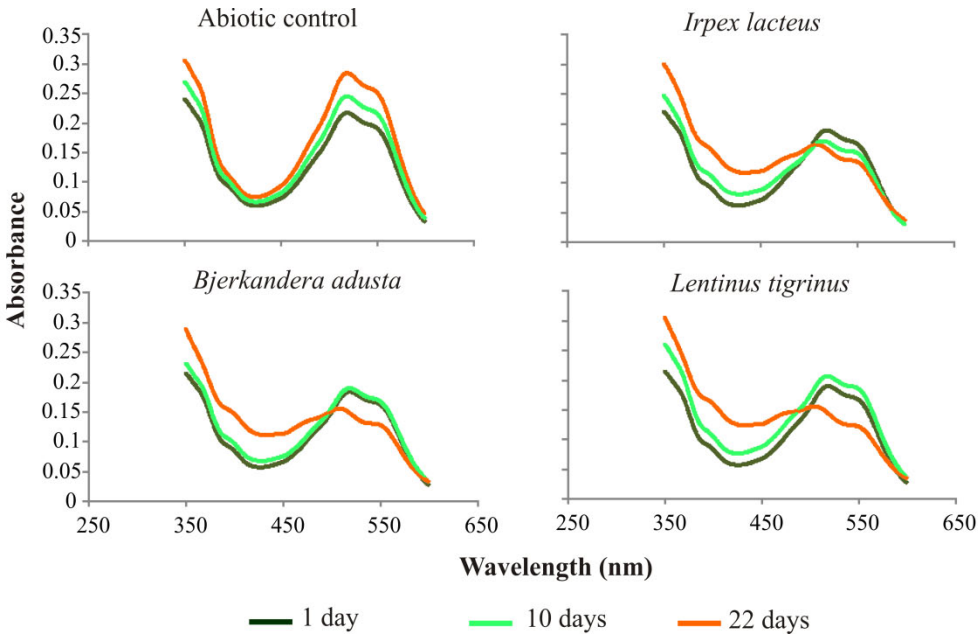


Figure 4.3 Absorbance spectra of Poly R-478 decolourization by three white-rot fungi and abiotic control after 1, 10 and 22 days of incubation in PAH containing liquid media with 10 mg/l of each PAH (dibenzothiophene, fluoranthene, pyrene and chrysene).

4.1.2 PAH degradation in 5 l slurry-phase reactor (II)

The scale-up of the soil slurry process was carried out with *Bjerkandera adusta* BOS55. Despite its apparent intolerance to seawater and its PAH degradation resembling the efficiency of the other two fungi, we selected *B. adusta* based on our previous knowledge of the fermentation pattern in stirred-tank reactors (Moreira et al., 2000a;

Moreira et al., 2000c). Moreover, previous studies with *B. adusta* BOS55 suggest that this fungal strain may be the ideal candidate to perform the slurry treatment of contaminated soil because of its capability to degrade PAHs under various conditions (Field et al., 1992; Kotterman et al., 1998) and to produce extracellular LMEs, especially MnP (Moreira et al., 2000a; Moreira et al., 2000c; Palma et al., 2000). To avoid any stress on the ligninolytic system of *B. adusta* due to salt concentration, inoculum and culture media were not prepared with sea water, but marsh soil and distilled water were used to perform all the slurry fermentations. The total concentration of the four PAHs (dibenzothiophene, fluoranthene, pyrene and chrysene) was 200 mg/kg, which corresponds well to actual contaminated soil.

Various operational parameters were assessed during the treatment of PAHs in slurry conditions: the inoculum type (pellets or mycelium), glucose concentration (10 - 23 g/l) and initial fungal biomass (0.64 - 2.2 g/l; Table 2 in II). *B. adusta* achieved a higher level of degradation of the four PAHs (48%) when applied as free mycelial suspension (Experiment B; Fig 4.4) than in the form of pellets (34%; Fig. 4.4: Experiment A). Pellets were only observed during the first seven days, indicating that the soil slurry conditions imposed a stress on the maintenance of the pellets inside the reactor (Fig. 4.5). Consequently, free mycelial suspension was selected as the inoculum type for the next experiments to evaluate the initial concentration of glucose and biomass on the degradation of PAHs (Fig. 4.4; Experiments B, C and D). The highest PAH degradation occurred in the slurry reactors inoculated with a maximal fungal biomass of 2.2 g/l and with an initial glucose concentration of 17 g/l (Fig. 4.4: Experiment C). Despite the fact that experiments C and D were duplicates, C had a lower initial glucose concentration (17 g/l) than D (23 g/l), which slightly enhanced the PAH degradation.

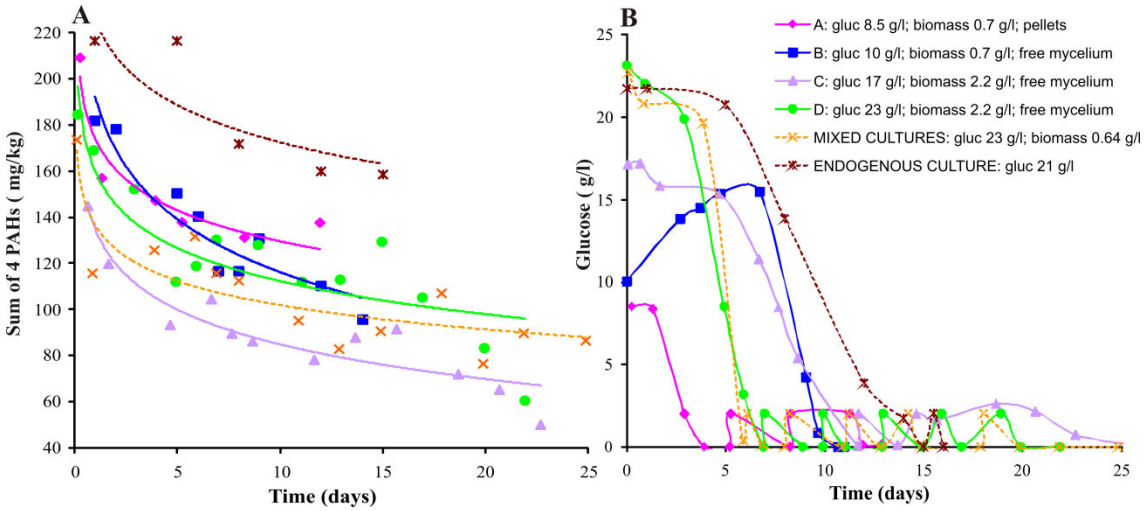


Figure 4.4 Degradation of four PAH compounds (average sum of dibenzothiophene, fluoranthene, pyrene and chrysene) and corresponding trend line (A), and glucose consumption (B) by *B. adusta* (experiment A-D), *B. adusta* + endogenous microflora (experiment "mixed cultures"), and solely endogenous microflora (experiment "endogenous culture") in 5 l slurry reactor at different operation conditions.

The degradation of PAHs by the fungus (Fig. 4.4: Experiments C and D with autoclaved soil) was compared to that from the abiotic control (data not shown), the biotic control (Fig. 4.4: Endogenous culture), and mixed cultures of endogenous microbes with *B. adusta*. The abiotic and biotic control incubations were carried out with autoclaved and non-sterile soil, respectively. The removal of the PAHs observed in the abiotic control was negligible, meaning that the PAHs did not evaporate or adhere to the reactor wall. Thus, the soil slurry experiment was designed correctly. When comparing the degradation profiles of endogenous microbes to fungal degradation (C and D), the results indicated that soil microbes had the potential to degrade PAHs, but that this potential was much more limited than that of *B. adusta* alone. *B. adusta* added to non-sterile soil (mixed cultures) showed a synergic action with soil endogenous microflora, especially in the initial 15 days of cultivation. After this, the final degradation of PAHs, especially in the case of fluoranthene and chrysene, was lower in the mixed cultures compared with that of the pure fungal cultures C and D (Fig. 3 in II). Synergistic effects have also been detected in *Bjerkandera* sp. strain BOS55 cultures enriched with forest soil, which enhanced PAH degradation by 20% after 215 days compared with the PAH degradation caused by the fungus alone (Kotterman et al., 1998). Thus, it could be possible that soil microbes also helped in breaking down the PAH metabolites.

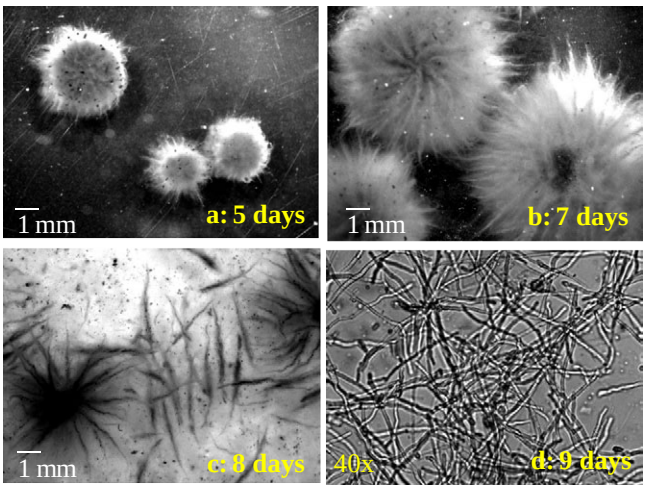


Figure 4.5 Pellet formation (a and b) and destruction (c and d) inside a 5 l agitated soil slurry reactor inoculated with *B. adusta*. Photos in panels a, b and c were taken using a magnifying glass. The photo in panel d was taken through a microscope 40x.

To assess the viability and performance of the mycelium, Poly R-478 plates were inoculated with samples taken from the reactor inoculated with *B. adusta*. The results showed that ligninolytic activity may be responsible for PAH degradation, as the plates turned from red to yellow during the whole experiment (Fig. 4.6). In contrast, neither decolourization nor typical *B. adusta* mycelium was observed on the incubated plates with mixed cultures (Fig. 4.7). Previous studies have shown that *B. adusta* produces two types of peroxidases, Mn^{2+}-dependent (MnP) and Mn^{2+}-independent peroxidase (MiP), with the ability to degrade PAHs (Palma et al., 2000; Wang et al., 2003). Under stirred conditions,

B. adusta produces MnP, the activity of which is not affected by agitation at any rate (Moreira et al., 2000a). Even in a similar contaminated soil slurry, *B. adusta* is able to produce MnP (Quintero et al., 2007). LiP production by *B. adusta* is induced by an organic N-rich medium (i.e., 5 g/l peptone) at a pH above 6.0 (Kaal et al., 1993). In this study, pH was constantly maintained at 4.5 - 5.0. Moreover, a peptone concentration of 2 g/l was added at pulses of 0.4 g/l every three days after its consumption, which indicated that N-limited conditions prevailed in the reactor. Thus, LiP was probably not produced. Since laccase activity has not been detected in cultures of *B. adusta* in either a N-limited or N-sufficient medium (Moreira et al., 1997; Schützendübel et al., 1999; Moreira et al., 2000b), MnP was likely the enzyme involved in PAH degradation.

Other slurry reactors have also been successfully applied to degrade organic contaminants (e.g., HCH, PCP and endocrine disruptor chemicals) by white-rot fungi (Quintero et al., 2007; Rubilar et al., 2007; Rodríguez-Rodríguez et al., 2010). From these results, and the results of this study, it can be concluded that a fungal slurry-phase reactor may be a good alternative for *ex situ* bioremediation of contaminated soil.

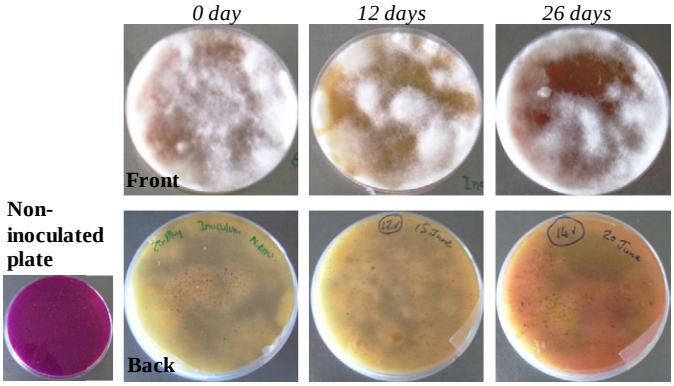


Figure 4.6 Growth and decolourization of Poly R-478 plates inoculated with samples from soil slurry fermentation of *B. adusta* (experiment C). Front = front side of the plate; Back = back side of the plate.

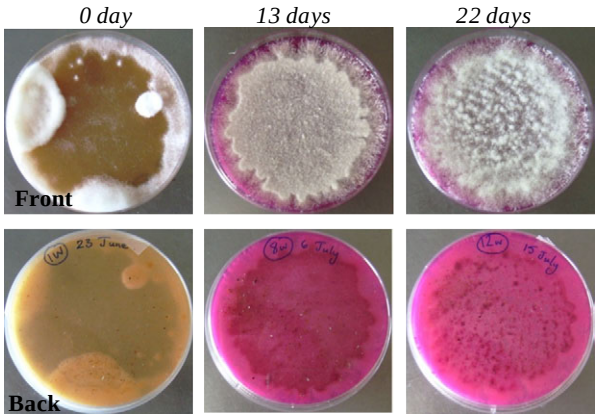


Figure 4.7 Growth and decolourization of Poly R-478 plates inoculated with samples from soil slurry fermentation of *B. adusta* + endogenous microflora (experiment "mixed cultures"). Front = front side of the plate; Back = back side of the plate.

4.1.3 Effect of soil slurry conditions in fungal growth and MnP activity (II)

Monitoring of the fermentation profile of *B. adusta* in a non-contaminated soil slurry (experiment C1 in II) showed that either the on-line operational parameters (pH, oxygen partial pressure and redox potential) or glucose concentration slightly changed during the first six days (Fig. 4.8). In a conventional liquid fermentation of *B. adusta* in the absence of soil, the lag period is shorter than in soil slurry (Moreira et al., 2000a; Moreira et al., 2000c). The extended lag phase may be explained by a limited oxygen supply due to the soil content in the slurry (Woo and Park, 1997). After the lag period, the fermentation profile of *B. adusta* in the soil slurry system was similar to that of conventional fermentation (Moreira et al., 2000c). The rapid glucose depletion on day seven coincided with the modification of the other operational parameters. At first, the pH increased from 4.7 to 6.0, followed by a rapid drop. This increase of pH may be due to the hydrolyses of peptone by proteases releasing ammonium, which is responsible for higher pH values in the reactor, as was reported previously (Moreira et al., 2000c). The redox potential initially decreased and it achieved a maximum peak when MnP activity was produced. The oxidation of Mn^{2+} to Mn^{3+}, a strong and very unstable oxidant, during the MnP cycle may explain the high oxidative condition created in the reactor from day eight to ten (Fig. 4.8). The fact that all these parameters changed simultaneously right before the onset of MnP production suggests that they may be used as indicators of MnP production.

The maximum MnP activity found in the soil slurry system was only 50 U/l (Fig. 4.8), which was lower than in conventional cultivations of *B. adusta* (200 U/l; Moreira et al., 2000a). Low production of MnP may be associated with the shear stress imposed by the presence of solid particles. This is in accordance with similar soil bioslurry systems in which fungal activity decreases as the solid content increases (Quintero et al., 2007; Rodríguez-Rodríguez et al., 2010). Regardless of the solid particles, various studies using liquid stirred-tank reactors have shown that an increase in the agitation speed suppresses the LMEs activities of various white-rot fungi, for example laccase produced by *Pleurotus ostreatus* (Kim and Song, 2009) or LiP produced by *Phanerochaete chrysosporium* (Michel et al., 1990).

The fungal growth morphology progressively changed from free mycelial suspension to the pellet shape typical of *B. adusta* in a conventional agitated fermentation at an agitation rate of 250 rpm (Moreira et al., 2000a). At day ten, the pellets had a clear dark core, suggesting that the fungus used soil as support for pellet formation (photos shown in II). Although few reports are available, enzyme production may be limited due to changes in the pellet's internal structure and to the compaction of the pellets (Niemenmaa, 2008; Kim and Song, 2009).

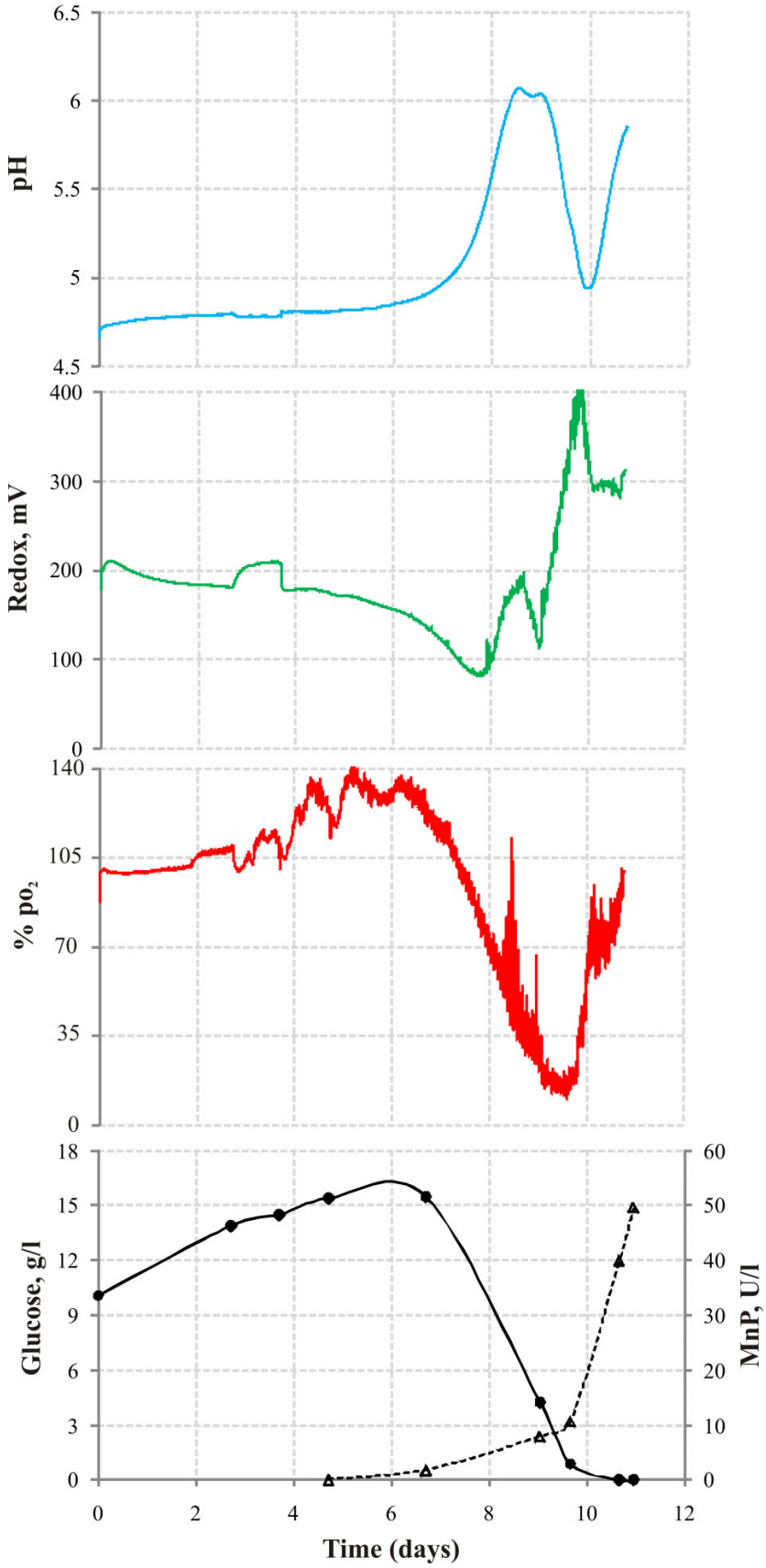


Figure 4.8 pH (—), redox potential (—), oxygen partial pressure (—), glucose concentration (●) and manganese peroxidase activity (▲) in function of time during incubation of *B. adusta* in a soil slurry-phase stirred-tank reactor.

4.2 Solid-phase pretreatment of contaminated soil (III and IV)

The applicability of fungal solid-phase pretreatment of contaminated soil was based on the intrinsic characteristic of fungi to degrade wood in nature and their ability to colonize soil. As with any other remediation technology, preliminary studies are necessary for the successful demonstration and development of the treatment process on a larger scale (Wilson and Jones, 1993). The experiments included several screenings of fungal species, treatability tests at various laboratory scales, and a scale-up of the process.

4.2.1 Screening assays (III)

Contaminant and soil tolerance is species specific; thus, the first selection of the best fungi within the group of wood-degrading basidiomycetes was based on the fungal tolerance to PCDD/F-contaminated soil and the ability of the fungi to compete against soil native microorganisms. In total, 146 fungal strains were screened, some of which were known to reduce the mass of wood considerably (Hakala et al., 2004), to cause serious damage to wood in nature (e.g., the dry-rot fungus *Serpula lacrymans*), or to tolerate and degrade chlorinated phenols in contaminated soil (Tuomela et al., 1999). The list of the tested fungi included 55 white-rot fungi (WRF), 12 brown-rot fungi (BRF), 52 litter-decomposing fungi (LDF), 10 fungi whose habitat overlaps with those of WRF and LDF (WRF-LDF), and 17 strains which were not identified (Annexed table shows full list of screened fungi).

In the first screening assay with non-sterile sawmill soil A (2.1 mg/kg I-TEQ PCDD/Fs and 9% organic matter), mostly LDF (28 strains out of 52) were able to grow and survive in contaminated soil (Annexed table). Among WRF, the growth of 13 strains out of 56 was not inhibited. With the exception of *Gloeophyllum sepiarium* and *Postia caesia*, no BRF were good soil colonizers. This secreening revealed 34 promising strains for the second screening with sawmill soil B (2.1 mg/kg I-TEQ PCDD/Fs and 84% organic matter). During the second screening, LDF were the most outstanding soil colonizers together with fungi whose typical habitat is in contact with soil but which also colonize wood (e.g., *Pholiota* sp.). Only a few WRF tolerated soil as well as LDF, with *Phanerochaete velutina* being one of the best white-rotters tested (Annexed table). In general, those fungi that showed extensive growth in the first screening did it also in the second one, confirming that organic matter has only a slight influence on the growth capability.

Both screening assays resulted in the selection of 18 fungal strains for subsequent assays of enzyme activity in agar-plates prepared with various indicators (results shown in Table 2 in III) and in solid-state cultivation on pine bark. Nearly all tested fungi had a positive reaction in the RBBR (14 strains), ABTS (14 strains), humic acids (13 strains) and CMC agar-plates (16 strains), suggesting that the fungi possessed extracellular production of oxidoreductases and cellulases. In the case of Mn-supplemented plates, the positive

reaction (i.e., formation of brown-black rings and spots of MnO_2) appeared only in the case of nine strains of fungi. This did not mean that the fungi lack MnP. The fungus *Sphaerobolus stellatus*, isolated from a sawmill area, which was negative in Mn-supplemented plates, showed a high activity of MnP (165 U/l) in liquid and in bark cultivations (Table 4.1). Results from the indicator agar plates did not provide any information about the most appropriate fungi for soil pretreatment.

Results from pine bark cultivations revealed that, out of 18 strains, at least half had clearly degradative activity (Table 4.1). Laccase activity was generally low and the activities of polysaccharide-degrading enzymes did not provide a sufficient selective tool due to low variation in the results. Thus, the selection criteria were MnP production and the mycelium extension in bark and soil, or a combination of these two results. Consequently, six strains of fungi were further investigated (Table 4.1)

These simple but thorough screening assays proved to be an efficient tool for selecting active fungi for further treatability tests. Other studies also show that positive results in indicator agar-plates coincide well with the enzyme activities in other media (Steffen et al., 2000; Rigas et al., 2003). However, there have only been a few screenings with non-sterile contaminated soil (Martens and Zadrazil, 1998; Meysami and Baheri, 2003). Usually studies have concentrated on tests without soil addition (Matsubara et al., 2006), neglecting that soil properties and endogenous organisms can suppress fungal growth and enzyme activity. Therefore, fungi that apparently are good candidates for pollutant degradation may be inefficient for bioremediation purposes. This work demonstrated that LDF actively colonized soil and bark and produced enzymes in these two media. These findings are also supported by other authors (Steffen et al., 2002a; Steffen et al., 2002c; Tuomela et al., 2005; Baldrian and Šnajdr, 2006; Osono, 2007; Šnajdr et al., 2008). Thus, preliminary screenings should also include LDF strains. Despite their apparent difficulty at growing in soil, the successful establishment of various WRF (particularly *P. velutina*) in this and other studies (Dowson et al., 1988a; Dowson et al., 1988b) leads to the conclusion that WRF are also promising candidates for fungal bioremediation applications, and thus, preliminary screening tests should include WRF as well.

Table 4.1 Fungal growth in contaminated sawmill soil B and in Scots pine bark, and enzyme activities in extracts from bark cultivated with various fungi after 20 days. Values show the means of three replicates ± standard deviation.

	Fungal growth		Enzyme activities in pine bark (mU/g of dry bark)[b]				
			Ligninolytic enzymes		Cellulases	Hemicellulases	
Fungus	Mycelium extension in contaminated soil[a]	Biomass in pine bark (mg/g of dry bark)	MnP	Laccase	Endo-1,4-β-glucanase	Endo-1,4-β-mannanase	Endo-1,4-β-xylanase
Agrocybe dura	+++	181	244 ± 79	61 ± 17	40 ± 18	18 ± 4	6 ± 0
Agrocybe praecox *	+++	248	695 ± 75	111 ± 89	19 ± 4	21 ± 9	9 ± 3
Collybia peronata	++	250	30 ± 27	8 ± 8	18 ± 10	13 ± 3	7 ± 3
Gymnopilus luteofolius *	+++	136	534 ± 247	6 ± 6	31 ± 6	41 ± 8	21 ± 3
Gymnopilus penetrans	++++	184	302 ± 146	9 ± 16	168 ± 38	20 ± 6	9 ± 3
Gymnopus erythropus	+	187	332 ± 59	331 ± 219	37 ± 2	27 ± 11	6 ± 3
Hypholoma fasciculare *	++	162	1511 ± 446	90 ± 157	76 ± 21	30 ± 8	14 ± 3
Kuehneromyces mutabilis	–	150	482 ± 51	111 ± 27	16 ± 1	17 ± 6	4 ± 1
Mycena galericulata	+	235	266 ± 45	231 ± 71	128 ± 11	19 ± 8	16 ± 1
Phanerochaete sanguinea	–	155	n.d. n.d.	58 ± 100	13 ± 2	16 ± 8	10 ± 1
Phanerochaete velutina *	++++	149	5258 ± 1783	2 ± 4	45 ± 11	135 ± 25	22 ± 2
Physisporinus vitreus	–	200	157 ± 49	408 ± 160	18 ± 3	18 ± 2	10 ± 2
Postia caesia	+	152	92 ± 3	n.d. n.d.	13 ± 1	22 ± 3	4 ± 0
Rhodocollybia butyraceae	++	162	2120 ± 362	31 ± 26	100 ± 17	29 ± 2	12 ± 2
Serpula lacrymans	–	260	88 ± 111	n.d. n.d.	14 ± 3	9 ± 3	2 ± 0
Sphaerobolus stellatus *	++++	113	1656 ± 197	67 ± 31	21 ± 2	26 ± 3	17 ± 2
Stropharia aeruginosa	+	180	87 ± 151	n.d. n.d.	19 ± 5	19 ± 0	11 ± 1
Stropharia rugosoannulata *	++	211	1261 ± 557	n.d. n.d.	59 ± 14	24 ± 4	33 ± 7

* Selected fungus for further experiments.

[a] Fungal growth: - negligible, + slight, ++ moderate, +++ intermediate, ++++ excellent.

[b] Statistical analyses are shown in Table 4 in III.

n.d. not detected.

4.2.2 Degradation of organic matter from various contaminated soils (III and IV)

The fungal degradation of OM as a pretreatment for contaminated soil has never been studied before. Thus, pretreatment studies at laboratory-scale using sawmill soils with a different OM content were necessary (sawmill soil A-D, see Table 3.2 for soil properties). First, the experiment was performed with six fungi and soil A (< 10% OM) using small-scale bottles (0.5 l). Then three fungi (*Gymnopilus luteofolius*, *Phanerochaete velutina*, *Stropharia rugosoannulata*) were later investigated with soil B (84% OM; Fig. 4.9). The same fungi plus *Sphaerobolus stellatus* were further tested at a medium laboratory-scale (2 l; results are shown in Fig. 2 in IV).

The respiration from bark-amended non-sterile sawmill soil A was similar to the fungal respiratory activity in bark (no soil addition; Table 6 in IV and Fig 4.9). This suggests that fungi, together with soil endogenous microbes, utilized mostly pine bark instead of soil OM. In contrast, a significant amount of CO_2 was evolved from bark-amended non-sterile sawmill B, which had an eight-fold higher OM than soil A, showing that fungi and microbes also degraded soil OM (Fig 4.9). Carney et al. (2007) also showed that an increased relative abundance of fungi led to more rapid rates of soil OM degradation, which correlated with the production of extracellular enzymes. This finding is also consistent with our study in which MnP and endo-1,4-β-glucanase activities from bark-amended sawmill soil B were significantly higher in cultures of *P. velutina* (Table 5 in IV), the most active fungus in terms of CO_2 production. Additionally, fungal respiratory activity was higher than that of soil endogenous microbes (Fig. 4.9). The latter produced more CO_2 when bark was added to soil (Fig. 4.9). Thus, the addition of bark may have stimulated endogenous microbes to consume OM. A possible priming effect, a phenomenon in which the degradation of soil organic matter is accelerated by the addition of organic materials in response to the increased activity of soil microbes (Kuzyakov et al., 2000), may likely have occurred. Blagodatskaya and Kuzyakov (2008) explained that a stimulation of fungal activity was the responsible priming effect and suggested that soil organic carbon degradation occurs as co-metabolism. In accordance with priming effect studies, two factors may have caused the stimulation of soil microbes: bark addition or fungal mycelium (Waksman et al., 1939). Studies with ^{14}C-labelled bark would have been necessary to prove whether the priming effect was due to the addition of the bark (Shen and Bartha, 1996; Kuzyakov et al., 2000; Tuomela et al., 2002).

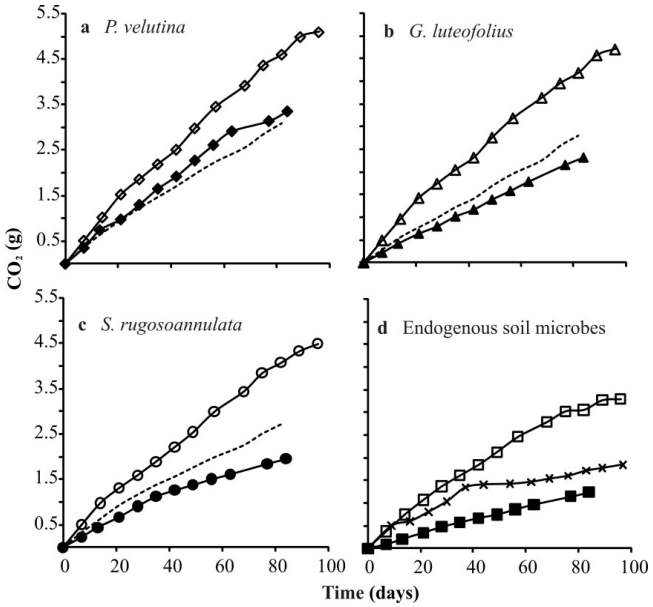


Figure 4.9 Respiratory activity of fungi (a-c) and soil endogenous microbes (d) in bark-amended sawmill soil A (◆, ▲, ●, ■,) and sawmill soil B (◇, △,○, □). Respiratory activity of corresponding fungi in autoclaved bark (- - - -) and of soil endogenous microbes in sawmill soil B without bark (×).

Soil organic carbon removal was calculated depending on the duration of each experiment and then carbon loss was estimated for six months (see equations in IV). This is the period when the average Finnish temperature is above +10 °C, the threshold value for fungi to grow in soil (Baldrian, 2008b). Table 4.2 shows soil carbon losses from fungal-treated sawmill soils with an OM higher than 80% at two bark:soil ratios: 21:100 and 14:100 (w/w). When considering the bark:soil ratio, the carbon loss in *P. velutina*-treated sawmill soils B and C was similar (6.4 and 5.3%, respectively). In contrast, a ratio of 14:100 of fungal bark inoculum to soil enhanced the carbon loss efficiencies of *G. luteofolius* (3.5 and 8.0%, respectively) and *S. rugosoannulata* (4.6 and 9.5%, respectively). The lack of experimental data prevented us from adequately explaining this fact, but one hypothesis may be that less added bark stimulated fungus to access soil OM. Another explanation may be that CO_2 production solely from bark without the presence of soil was suppressed as a result of inadequate liquid inoculation and this caused us to overestimate the actual soil carbon loss.

S. stellatus achieved the highest CO_2 production from sawmill soil (Table 2 in IV), a result which corresponds to 20% of organic carbon removal in six months. This fungus was isolated from a former contaminated sawmill site, which may explain its adaptability to the contamination. During the cultivation process, *S. stellatus* produced dark-coloured spores on the top of the soil and on the soil/flask wall interface. At a temperature of 15 - 20 ºC this fungus ejects highly resinous spores for a distance of several meters, causing abrasive damage to different materials, such as vinyl capstocks (Grossman, 2005). Spreading such spores during the bioremediation process would not be desirable, thus *S. stellatus* was omitted from further experiments.

Table 4.2 Total CO_2 production in bark-amended sawmill soil B and C (CO_2 soil + bark) at two scales (0.5 and 2 l) by *G. luteofolius*, *P. velutina* and *S. rugosoannulata* and in autoclaved bark (CO_2 bark). The estimated soil organic carbon loss for six months was used to calculate the combustion cost after pretreating the soil.

	Sawmill soil B - 84% (OM) 48% (OC)[a] Bark:soil 21:100 Scale 0.5 l			**Sawmill soil C - 82% (OM) 46% (OC)[a] Bark:soil 14:100 Scale 2 l**		
	G. luteofolius	*P. velutina*	*S. rugosoannulata*	*G. luteofolius*	*P. velutina*	*S. rugosoannulata*
Time (days)	96	96	96	84	90	70
CO_2 soil + bark (g)	4.7	5.1	4.5	22.2	25.9	23.5
CO_2 bark (g)	4.1	4.0	3.7	7.0	17.6	4.2
CO_2 soil (g)	0.6	1.1	0.8	15.2	8.3	19.3
Calculated soil C loss (%)[b]	1.8	3.4	2.4	3.8	2.1	4.8
Soil OC loss in 6 months (%)	3.5	6.4	4.6	8.0	5.3	9.5
Soil OM in 6-month fungal pretreatment (%)[c]	81	79	80	75	78	74
Combustion cost before fungal treatment (€/ton)	134	134	134	132	132	132
Combustion cost after 6-month fungal treatment (€/ton)[d]	131	129	130	125	128	124

[a] OM = organic matter; OC = organic carbon; OM/OC for soil B = 1.75; OM/OC for soil C = 1.78.
[b] Equations are showed in publication IV.
[c] Calculated taking into account the corresponding OM/OC for each soil (see a).
[d] Calculated with the linear equation: Y (€/ton) = X (%) + 50, where Y is the combustion cost per ton and X the percentage of soil OM.

Although *G. luteofolius*, *P. velutina,* and *S. rugosoannulata* showed promising soil carbon losses, cost calculations were needed to estimate the efficiency of fungal pretreatment for contaminated soil prior to combustion. In Finland, the most common methods for treating contaminated soils are *ex situ* methods, and if the fill-up of landfills is excluded, the utilization of treated soils is negligible (Jaakkonen, 2008). In addition, composting is generally the best method to treat organic pollutants (mainly petroleum hydrocarbons) and combustion at high temperatures for heavily contaminated soils with persistent organic pollutants, such as PCDD/Fs (Pajukallio, 2006). Often, this method encounters problems related to soil properties. As an example, the combustion cost of heavily contaminated soil can range from 90 € to 150 € per ton depending on the soil OM content (Rantsi R., Niska & Nyysönen Ltd., personal communication). Since information on cost variation depending on the soil OM content is not available, the following scenario is hypothesized: if soil OM accounts for less than 50%, the combustion cost would be ≤ 90 € per ton since fuel consumption would be lower. If soil OM accounts for more than 50%, the cost would increase at the rate of 1 €/ton per percentage unit of OM following a linear equation (Eq. 4.1).

$$\text{Eq. 4.1:} \quad Y\ (€/\text{ton}) = X\ (\%) + 50$$

where Y is the combustion cost and X the percentage of soil organic matter. According to Eq. 4.1, the cost to combust one ton of sawmill soil B (84% OM) would be 134 €/ton and for soil C (82% OM) 132 €/ton. A six-month fungal pretreatment of soil would diminish the total combustion costs by 3 - 8 € per ton depending on the applied fungus (Table 4.2). Considering that in Finland there are approximately 200,000 tons of heavily contaminated soil (Järvinen and Salonen, 2004) which will likely be combusted, and hypothesizing that soil OM will account for more than 50%, the total gross savings would be 0.6 - 1.6 million euros if this fungal pretreatment method was to be applied before combustion.

The cost of the fungal bioremedation is not available as the technology has yet not been commercially implemented. The main costs affecting the total cost of the fungal technology, excluding the cost of soil excavation and transportation, will be derived from the production of liquid fungal inoculum in fermentors, the lignocellulosic substrate, the pre-conditioning of the substrate (e.g steaming) and the production of the inoculum to be introduced in the soil. Additional factors which may raise the cost are for example preliminary treatability tests, aeration of the pile, and monitoring of the remediation process. Considering all the costs, the technology will be feasible as a remediation technology as such, rather than a pretreatment. To become a cost-competitive method, the fungal bioremediation cost should be similar to that of biopiles or composting, which in Finland is around 8-60 €/ton depending on the type of contamination and the time needed for treatment (Vik and Bardos, 2002).

4.2.3 Scale-up of the fungal pretreatment process (IV)

The development and scale-up of the fungal pretreatment process was carried out with *Stropharia rugosoannulata*, a litter decomposing fungus with an excellent ability to colonize non-sterile soils, to produce dense mycelium and to degrade soil OM. The capacity of *S. rugosoannulata* to degrade PAHs in soil and to produce MnP were also reasons for selecting it (Steffen et al., 2000; Steffen et al., 2007).

Specific mesh plastic tubes for delivering fungal-bark inoculum to the soil prevented an extra load of OM from the bark into the soil. They were 70 cm long and filled with bark chips inoculated with fungal mycelia (Fig. 4.10 a). From the bark-filled tubes, *S. rugosoannulata* formed extensive mycelia (Fig. 4.10 b) ramifying to the surrounding soil (photo in Fig. 4.11). Like mycelia-forming basidiomycetes in soil (Cairney, 2005), *S. rugosoannulata* was able to proliferate and relocate mycelia with exploratory fan-shape in search for new nutrient resources. This observation demonstrated the ability of this litter-decomposing fungus to tolerate non-sterile contaminated soil. Grazing by soil-inhabiting invertebrates (particularly nematodes) can result in a dramatic loss of mycelium. In fact, *S. rugosoannulata* and the white-rot fungus *P. velutina*, which was the only white-rot fungus growing extensively in the soil, have showed nematode-attacking activity (Luo et al., 2006; Wood et al., 2006).

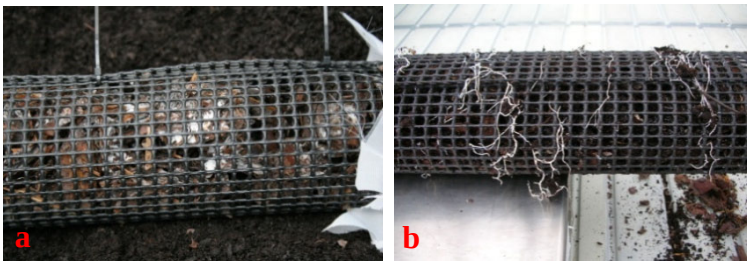


Figure 4.10 Fungal mesh tube before introduction to soil (a) and with well-developed mycelium of *Stropharia rugosoannulata* after 173 days (b).

Respiratory activity and the mass loss rate along the treatment of sawmill soil D (82% OM) can be divided into three stages (Fig. 4.11):

i) Stage 1 (0 - 14 d): Initial establishment of the fungus in the soil led to elevated CO_2 production and a sharp increase in the soil mass loss rate. The absence of fungal mycelium suggested a stimulation of soil endogenous microbes, likely due to the supplied aeration, resulting in the degradation of labile organic carbon (Šantrucková et al., 2004).

ii) Stage 2 (14 - 70 d): Mycelium developed progressively until day 70 (photo in Fig. 4.11), when the growth ceased along with the decrease of mass loss rate and CO_2 production. At this stage, the fungus and soil native microbes concomitantly degraded organic carbon (Kuzyakov et al., 2000).

iii) Stage 3 (70 - 173 d): Mass loss rate and respiration activity remained constant at 0.06%/day and 0.3 g of CO_2/day, respectively. Despite the apparent weak mycelial growth observed through the Plexi-glass window, the mycelium strongly colonized the soil and wood particles. The final net mass loss due to the degradation of the soil OM was 30.5 kg, which represents 10% of the original soil mass (308 kg).

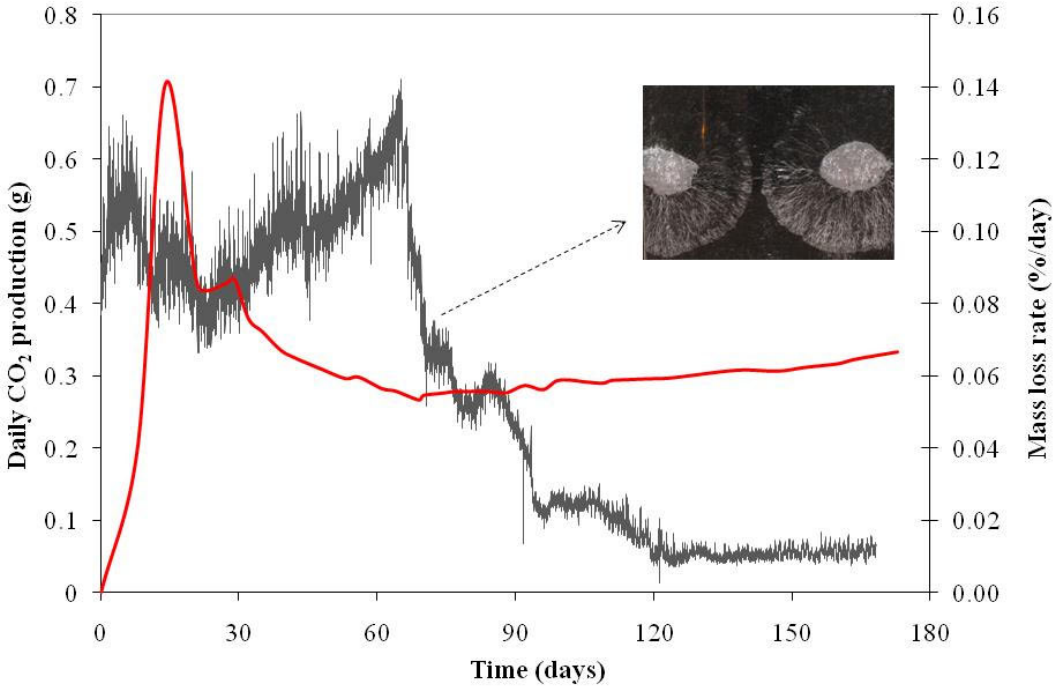


Figure 4.11 Daily CO_2 production (—) and soil mass loss rate (—) during pretreatment of sawmill soil D with *Stropharia rugosoannulata* in a large scale reactor (0.56 m^3).

4.3 Scots pine bark as lignocellulosic substrate for fungi (V)

4.3.1 Scots pine bark composition

Regardless of the tree species, bark is generally characterized by a higher content of extractives and lignin than the corresponding stemwood (Fengel and Wegener, 1989a; Fradinho et al., 2002). In this study, chemical characterization of Scots pine (*Pinus sylvestris*) bark demonstrated that the lignin content was 45%, about two-fold higher than that of the corresponding wood (Fengel and Wegener, 1989b). The content of cellulose was higher than that of hemicellulose (25.4% and 14.7%, respectively; Table 4.3), which is common in conifer bark (Fengel and Wegener, 1989a). Total extractives (water extractives and aqueous acetone extractives) accounted for 19.3% of the total dry mass. The composition of lipophilic extractives is available for Scots pine wood (Martínez-Íñigo et al., 1999; Dorado et al., 2000; Dorado et al., 2001; Willför et al., 2003). From bark, however, only the hydrophilic extractives (e.g., lignans, tannins, and flavonoids) are known (Pan and Lundgren, 1996; Karonen et al., 2004a; Karonen et al., 2004b; Sinkkonen et al., 2006). Thus, we only focused on the characterization of lipophilic extractives. Of these extractives, the most abundant compounds were as follows (Table 4.3, Fig. 4.12 and Fig. 4.13):

i) As in coniferous wood (Martínez-Íñigo et al., 1999), resin acids were the most abundant fraction of pine bark; and from these dehydroabietic acid was the major compound.

ii) The second major fraction corresponded to the group of sterols, of which the concentration of sitosterol was higher than in the corresponding wood: 176 mg/100 g and. 20 mg/100 g, respectively (Dorado et al., 2000).

iii) The phenolic acid 3,4-dihydroxybenzoic acid (30 mg/100 g).

iv) The unsaturated fatty acids (UFA) oleic (C 18:1 n-9), linoleic (C 18:2) and linolenic (C 18:3) acids, which are also the most abundant UFA in the corresponding wood (Dorado et al., 2000).

v) The saturated fatty acid behenic acid (C 22:0).

Table 4.3 Polysaccharides, lignin and aqueous acetone (95:5; v/v) extractives in Scots pine (*Pinus sylvestris*) bark before and after 90 days of incubation with *Phanerochaete velutina* or *Stropharia rugosannulata*.

	Bark before incubation	**Bark degraded by *P. velutina* after 90 d**	**Bark degraded *by S. rugosoannulata* after 90 d**
Polysaccharide (mg/g of dry bark)			
Cellulose[a]	254	54	164
Glucose (as free sugar)	0.29	0.15	0.10
Cellobiose (as free sugar)	0.02	0.29	0.13
Hemicellulose[a]	147	51	65
Lignin (mg/g of dry bark)[b]	449	388	382
Aqueous acetone extractives (mg/100 g of dry bark)			
Phenolic acids, aldehydes, dicarboxylic acids	44	18	14
Unsaturated fatty acids	123	17	15
Saturated fatty acids	83	120	82
Resin acids	447	233	248
Oxidized resin acids	87	79	50
Sterols	278	208	254

[a] Cellulose and hemicellulose contents are relative to the neutral detergent fibre (NDF) of bark.
[b] Lignin content shows only Klason lignin because acid soluble lignin accounted for less than 1% of the total lignin content.

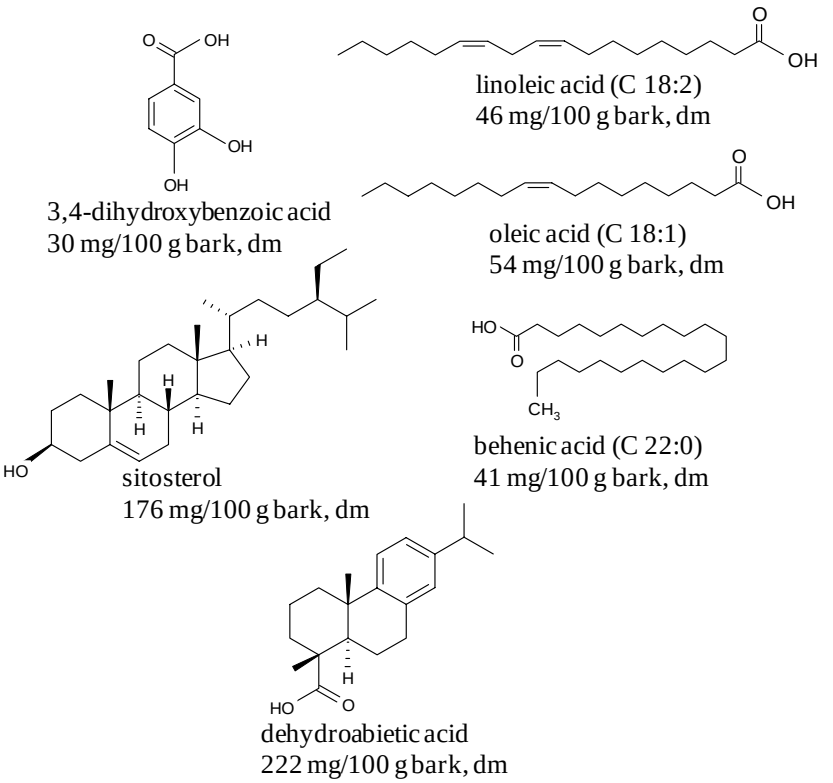


Figure 4.12 Chemical structure and concentration of the most abundant lipophilic extractives found in Scots pine (*Pinus sylvestris*) bark.

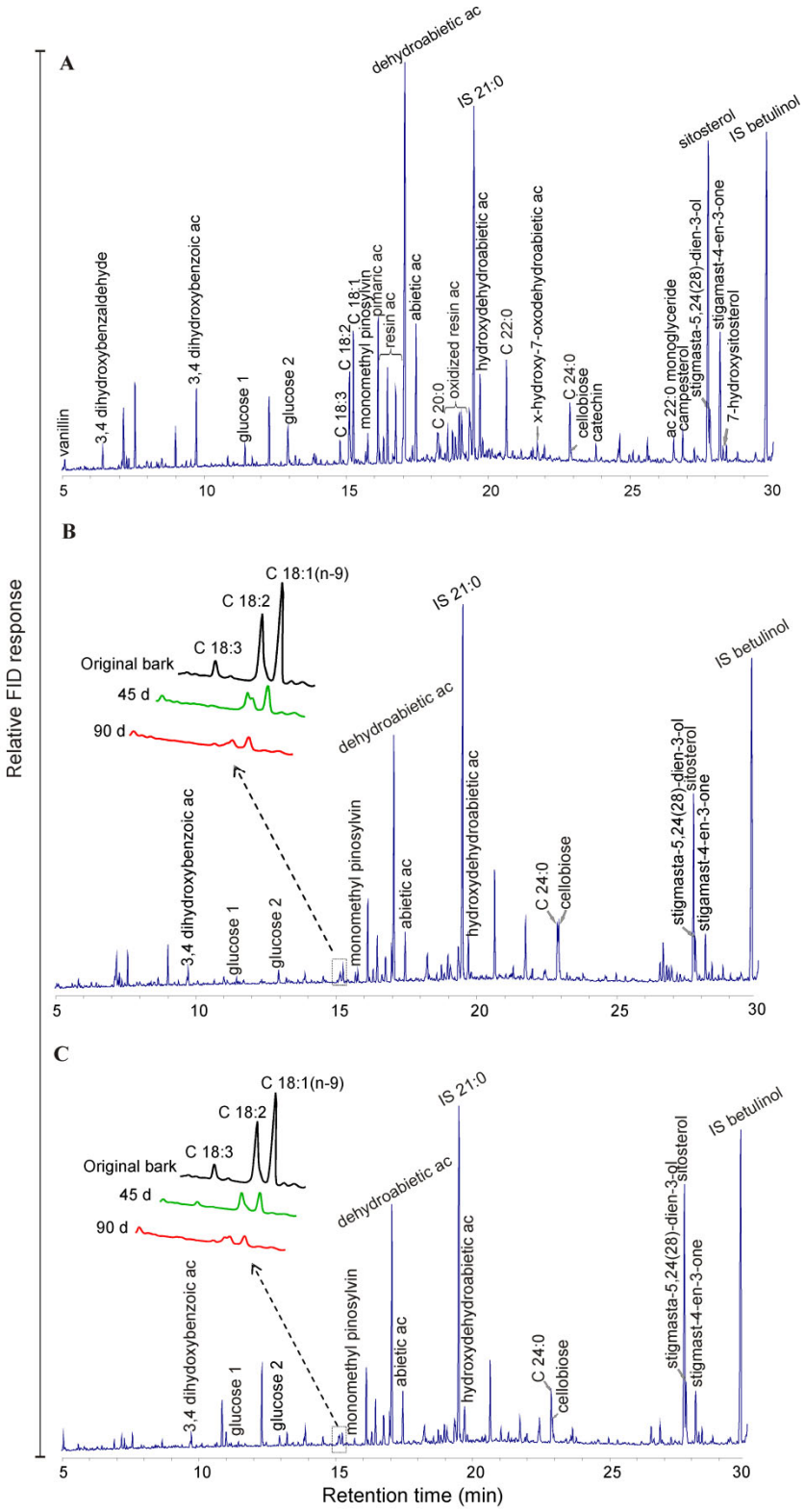


Figure 4.13 GC-MS chromatograms of the acetone extractives in the original Scots pine bark (A) and after a 90-day cultivation period with *Phanerochaete velutina* (B) and *Stropharia rugosoannulata* (C), and a detailed section (as insert in B and C) showing the modifications of unsaturated fatty acids oleic acid (C 18:1), linoleic acid (C 18:2), linolenic acid (C 18:3) at retention times between 14.5 and 15.5 min. The internal standards (IS) were henecosanoic acid (21:0) and betulinol.

4.3.2 Fungal degradation of pine bark

4.3.2.1 Degradation of polysaccharides and lignin

The analyses of polysaccharides demonstrated a similar degradation pattern in cellulose and hemicellulose for both fungi cultivated in bark (Fig. 4.14; hemicelluloses degradation in Table 3 in V). In the first stage, glucose accumulated during 15 or 45 days in bark inoculated with *S. rugosoannulata* and *P. velutina*, respectively (Fig 4.14 A). Depolymerization of hemicellulose likely led to the increase in glucose since cellulose degradation started later: after the first 15 days for *S. rugosoannulata* and 45 days for *P. velutina* (Fig. 4.14 B and Table 3 in V). In the second stage, *P. velutina* caused constant cellulose loss, reaching 80% after 90 days (Fig 4.14 B). On the contrary, cellulose was less readily degraded by *S. rugosoannulata* and only a 35% loss was attained (Fig. 4.14 B). For both fungi, cellobiose increased simultaneously as cellulose was depolymerized (Fig 4.14 B). This coincided with an increase in the cellulase (endo-1,4-β-glucanase) activity, showind the role of this enzyme in bark cellulose degradation (Fig 4.14 C). The highest production of the hemicellulase endo-1,4-β-mannanase at the beginning of incubation (Fig. 2 in V) showed a correlation with the initial degradation of hemicellulose (Table 3 in V). But later the low activity of hemicellulases could not explain the subsequent hemicellulose loss.

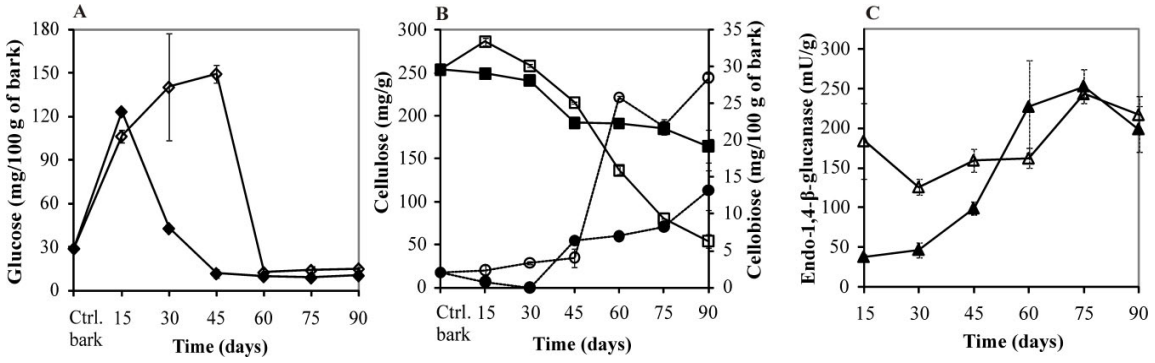


Figure 4.14 Concentration of glucose (A; ◆, ◇), cellulose (B; ■, □) and cellobiose (B; ●, ○), and activity of the cellulase endo-1,4-β-glucanase (C; ▲; △) in function of time during incubation of *Phanerochaete velutina* (◇, □, △) and *Stropharia rugosoannulata* (◆, ■, ▲) in Scots pine bark. Ctrl. bark = original non-inoculated bark.

Lignin was progressively degraded during the incubation of bark with both fungi, which after 90 days attained similar lignin loss (14%). Hemicellulose loss was, however, slightly higher with *P. velutina* (65%) in comparison with *S. rugosoannulata* (56%; the time course degradation of lignin and hemicelluloses is shown in Table 3 in V). Overall, the degradation profiles of polysaccharides and lignin indicated that the studied fungi were non-selective strains (i.e., the fungi simultaneously attacked all the components of bark). Non-selective wood-degrading fungi are probably more interesting for bioremediation than selective ones. Non-selective fungi may produce a wider array of extracellular oxidative

and hydrolytic enzymes essential for fungal survival in soil and for degradation of organic contaminants (Baldrian, 2008b).

4.3.2.2 Modification of extractives

Resin acids and oxidized resin acids are the main defense of coniferous trees against microbial attack (Savluchinske-Feio et al., 2006). Although pine bark had an extraordinarily high content of dehydroabietic acid (0.22%, w/w), the fungal growth was apparently not inhibited. Quite the contrary, 90 days of fungal treatment caused substantial degradation of dehydroabietic acid (Fig 4.13 and Table 2 in V): 42% by *P. velutina* and 44% by *S. rugosoannulata*. Polar hydroxyl-dehydroabietic acids are the products of the biotransformation of dehydroabietic acid by other wood-degrading fungi (van Beek et al., 2007). In contrast to the situation in aquatic ecosystems (Liss et al., 1997), resin acids and their byproducts have a limited toxicity to soil microbes (Fraser et al., 2009), which is an important fact if pine bark is used as fungal substrate for bioremediation.

The removal of the main sterol, sitosterol, varied between the two studied fungi. The white-rot fungus *P. velutina* degraded sitosterol up to 24%, whereas the litter-decomposing fungus *S. rugosoannulata* caused only 6% removal (Fig. 4.15 A). Compared with sapstain fungi (Martínez-Íñigo et al., 1999), litter-decomposing fungi may have only a limited ability to degrade sterols of wood and bark, but more studies with other litter-decomposing fungi are needed to confirm this. Martínez-Íñigo et al. (1999) reported the degradation of sitosterol in Scots pine wood by the white-rot fungus *Bjerkandera* sp. In comparison with bark, sitosterol removal is similar to that of heartwood (18%; Martínez-Íñigo et al., 1999), whereas the fungus causes higher sitosterol removal from sapwood (69-100%; Martínez-Íñigo et al., 1999; Dorado et al., 2000). Sapwood is, in general, more susceptible to microbial degradation due to its higher permeability to water and oxygen, and its lower concentration of toxic compounds in comparison with heartwood or bark (Eaton and Hale, 1993). In addition to sitosterol, other sterols also constituted bark: (iso-) fucosterol (stigmasta 5,24(28)dien-3-ol) and the steroid ketone stigmast-4-en-3one (Fig. 4.13 and Fig. 4.15 B). The fungal incubation of bark led first to an increase of the steroid ketone, the content of which was progressively decreasing after 30 days (Fig. 4.15 B). As Martínez-Íñigo et al. (2000) indicate, the steroid ketone may be an intermediate product of the fungal degradation of sitosterol due to the action of sterol oxidases. In addition, the increase in (iso-) fucosterol may be explained by the capacity of fungi to hydrolyze sitosterol and convert it to fucosterol, but more studies are needed to confirm this.

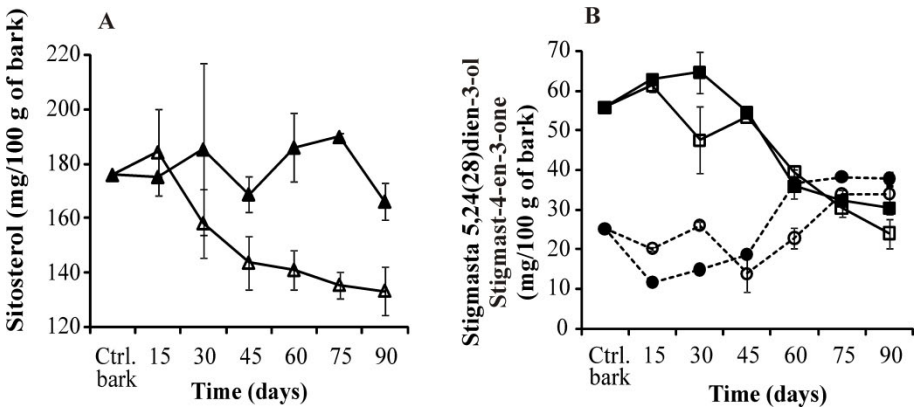


Figure 4.15 Concentration of sitosterol (A; ▲, △), the steroid ketone stigmast-4-en-3-one (B; ●, ○) and (iso-) fucosterol (stigmasta 5,24(28)dien-3-ol) (B; ■, □) during incubation of *Phanerochaete velutina* (△,○,□) and *Stropharia rugosoannulata* (▲,●,■) in Scots pine bark. Ctrl. bark = original non-inoculated bark.

P. velutina and *S. rugosoannulata* caused similar losses of the UFA oleic (83% and 86%, respectively), linoleic (89% and 90%, respectively) and linolenic acids (96% and 90%, respectively) after 90 days of treatment (Fig, 4.13 and Table 2 in V). The degradation of total UFA in bark was similar (86% by *P. velutina* and 88% by *S. rugosoannulata*) to the corresponding heartwood (Martínez-Íñigo et al., 1999), but lower than for sapwood degradation by various white-rot fungi (Dorado et al., 2000; Dorado et al., 2001). This confirms the resistance of bark to degradation. The resistance of bark from various trees against fungal attack is due to the inhibiting effect of suberin and tannins on the fungal growth (Kostov et al., 1991; Vane et al., 2006) or on the enzyme activities (Scalbert, 1991). The slow degradation of bark may be an advantage for bioremediation applications since bark will constantly supply to fungi the components necessary for growth and promotion of the degradative capacity of enzymes to treat contaminants (Hofrichter et al., 2001).

During fungal cultivation on pine bark, the production of MnP activity coincided with the highest degradation rate of UFA and lignin loss (Fig. 4.16). Phenolic lignin substructures may have been degraded via diffusible Mn^{3+}-chelator complexes (Hofrichter et al., 2010), but destruction of non-phenolic lignin substructures must occur by other mechanisms. Reactive peroxyl radicals originating from UFA peroxidation by MnP-mediated activity are responsible for the oxidation of non-phenolic β-*O*-4-linked lignin model compounds (Kapich et al., 1999a; Kapich et al., 1999b; Kapich et al., 2005). Kapich et al. (1999a) observed that highly unsaturated fatty acids enhance lignin destruction. In this study, fungi degraded more di- and tri- unsaturated fatty acids, that is, linoleic and linonelic acids, than the monounsatured oleic acid. It is possible that when using pine bark in bioremediation as a lignocellulosic substrate rich in UFA, the MnP-mediated lipid peroxidation process may contribute to the degradation of organic contaminants in the soil.

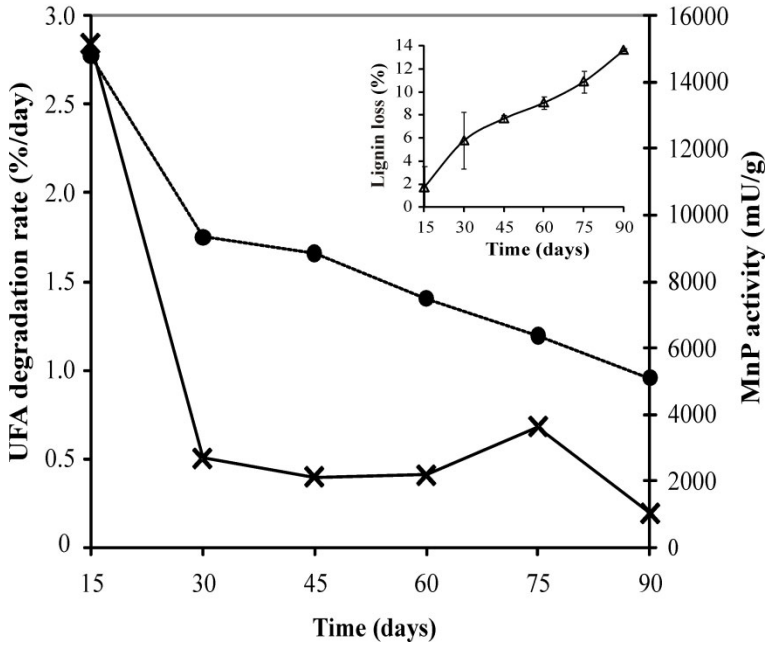


Figure 4.16 Total degradation rate of unsaturated fatty acids (UFA; ●) and manganese peroxidase (MnP) activity (×) during the incubation of *Phanerochaete velutina* in Scots pine bark. Inserted small panel shows the cumulative lignin loss.

5 CONCLUSIONS

This study describes the potential application of two novel *ex situ* fungal technologies. The first part of this thesis evaluated the degradation potential of white-rot fungi in a **slurry-phase reactor** to treat PAH-contaminated marsh soil. The second part evaluated the applicability of fungi in **solid-phase pretreatment** to degrade organic matter from contaminated soil, as well as Scot pine (*Pinus sylvestris*) bark, as a lignocellulosic substrate for fungal bioremediation. The following conclusions can be derived from the results:

Soil slurry-phase reactor

- Under saline conditions, only a few white-rot fungi exhibited high levels of degradation of PAHs. The most halotolerant fungi were *Lentinus tigrinus* PW93-4, *Irpex lacteus* Fr. 238 617/93 and *Bjerkandera adusta* BOS55.

- *Lentinus tigrinus* and *Irpex lacteus* exhibited a Poly R-478 decolourization ability in a medium prepared with 100% sea water, indicating that their ligninolytic activity was not hindered.

- The scale-up of the slurry-phase technology from a 100 ml reactor to a 5 l reactor was successfully developed using *B. adusta*.

- In the 5 l slurry reactor (10% soil concentration and 250 rpm) *B. adusta* achieved a maximum PAH degradation with: i) inoculum in the form of free mycelium, ii) 2.2 g/l fungal biomass and iii) a 17 g/l initial glucose concentration. Degradation also occurred with *B. adusta* in cooperation with endogenous soil microbes.

- The fermentation profile of *B. adusta* in the slurry soil system was similar to that of conventional fermentation, but with a lag phase of MnP production and glucose consumption of six days.

Solid-phase pretreatment

- From the evaluated fungal ecophysiological groups, mainly litter-decomposing fungi, with the exception of the white-rot fungus *Phanerochaete velutina*, exhibited extensive growth in non-sterile contaminated soil.

- The main enzymes that fungi produced in bark and soil were manganese peroxidase (MnP) and endo-1,4-β-glucanase.

- During fungal pretreatment, depending on the applied fungus, soil organic carbon decreased by 3.5% - 9.5% over the course of a six-month incubation period.

- Fungal pretreatment technology to decrease soil organic matter was successfully developed and scaled-up using *Stropharia rugosoannulata*, which attained 10% of soil mass loss

- Pre-inoculated pine bark was filled into mesh plastic tubes and introduced to the soil. These bark-filled tubes prevented an extra load of organic matter. From these tubes, the fungus colonized the surrounding contaminated soil.

Scots pine bark as substrate for fungal inoculum

- The main lipophilic compounds of Scots pine bark were dehydroabietic acid, sitosterol, 3,4-dihydroxybenzoic acid, unsaturated oleic, linoleic and linolenic acids, and saturated behenic acid. Pine bark contains more lignin (45%) than cellulose (25%) or hemicellulose (15%).

- The production of MnP coincided with the degradation rate of unsaturated fatty acids and lignin loss. MnP-mediated lipid peroxidation likely played a role in lignin degradation. MnP-initiated lipid peroxidation may be beneficial for the degradation of contaminants.

- Scots pine bark was an ideal lignocellulosic substrate for the growth of basidiomycetes and production of extracellular enzymes. At the same time, pine bark prevented the growth of undesirable microbes (e.g., moulds) and provided a slow but continous source of nutrients to fungi.

6 FUTURE APPLICATIONS

Bioremediation is a sustainable technology for treating contaminated soil with minimal environmental impact and low resource consumption. Despite the large potential of this technology, landfilling and stabilization still prevail in the market. In 2009, the European Commission launched an initiative to promote novel technologies with an emphasis on sustainable and cost-efficient remediation technologies such as bioremediation (Commission of the European Communities, 2004). Traditionally, bioaugmentation processes apply bacteria. Low specificity towards the contaminant type, a hyphal growth mode and the production of extracellular enzymes are the advantages that fungi possess to be applied in bioremediation. This thesis successfully developed two novel fungal *ex situ* bioremediation technologies and introduce new insights for their further full-scale application.

The first part of the thesis showed that if a soil slurry-phase reactor is the method adopted for an *ex situ* treatment in marine sites, it is advisable to prepare the slurry without sea water, as the salt concentration may affect the capability of fungi to degrade pollutants. The application of a fungal slurry-phase reactor may treat a wide range of organic contaminants without the need to autoclave the soil. There are, however, factors which might hinder reactor efficiency at full scale: 1) if the soil is not sieved, large rocks might damage the reactor; 2) soil watering, as well as wastewater post-treatment, might make the application too expensive. Nevertheless, fungi may be applied for removal of pharmaceutical and personal care products (PPCPs) and other emerging pollutants (e.g., illicit drugs and surfactants) found in effluents and sludge from wastewater treatment plants (WWTPs). Conventionally activated sludge technologies are unable to remove them, and there are, consequently, a constant release of PPCPs into the environment. PPCPs are often aromatic; thus, due to their unspecific enzyme system, fungi might be applied in bioreactors to clean up the effluents and sludge from WWTPs.

In the second part of the thesis, a solid-phase pretreatment was designed as an *ex situ* technology to degrade soil organic matter. Considering the successful development of the technology, the extensive growth of fungi in soil and their potential to degrade recalcitrant organic contaminants, this technology might be also applicable as *in situ* bioremediation of contaminated soil. Figure 6.1 shows a proposed *in situ* bioremediation for PAH-contaminated soil in which fungal tubes are vertically arranged. The tubes might be replaced, when necessary, thereby providing a fresh fungal bark inoculum. The fungus will first colonize the bark and progressively produce new mycelium from the tube to the surrounding soil. While colonizing the soil, the fungus produces extracellular lignin-modifying enzymes, which, in cooperation with soil microbes, have the capability to mineralize the contaminants. To enhance fungal growth, an adequate air supply might be required (Fig. 6.1). The challenges to perform this technology at large scale will depend on the specific site properties, the contaminant and the acclimatization of the fungus to the

environmental conditions. For this, it would be advisable to perform pre-treatability tests at laboraty scale to select the most suitable fungal strain and, thus, to estimate the degradability level of the contaminant.

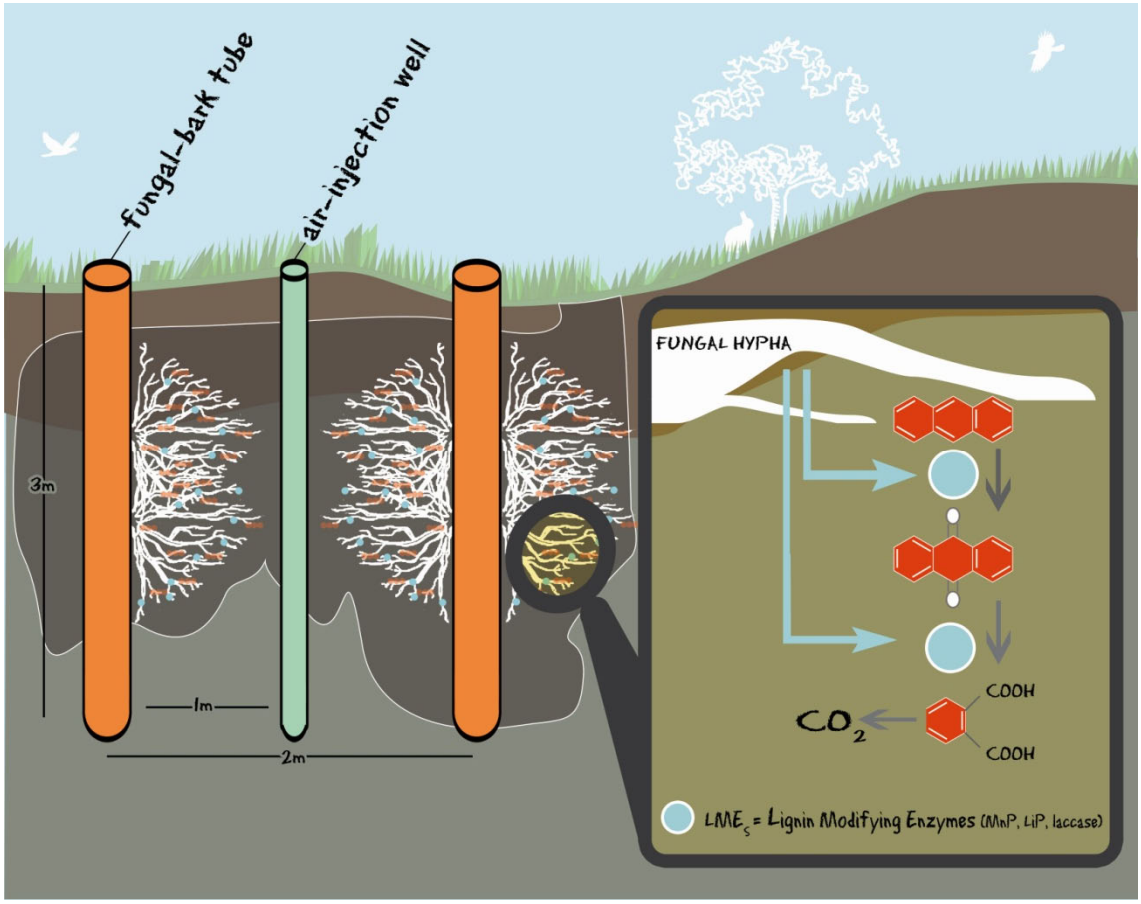


Figure 6.1 Schematic illustration of a hypothetical *in situ* bioremediation of PAH-contaminated soil with fungal-bark tubes and aeration wells. Illustration by Roberto Valentín.

Annexed table: Screened fungi for the solid-phase pretreatment of contaminated soil.

	Group	Fungus	Strain	Former code	Growth in sawmill soil[a]	
					A	**B**
1	**LDF**	***Agrocybe dura ****	FBCC478	MW 71-2	+++	+++
2	LDF	*Agrocybe praecox*	FBCC587	K163	++	+++
3	**LDF**	***Agrocybe praecox ****	FBCC476	TM 70.84 (A70-1)	+++	+++
4	WRF	*Amylostereum chailletii*	FBCC577	K150i	–	
5	BRF	*Antrodia serialis*	FBCC106	308, PO12	–	
6	WRF	*Antrodiella parasitica*	FBCC518	K43	+	
7	WRF	*Bjerkandera adusta*	FBCC300	213	–	
8	WRF	*Bjerkandera adusta*	FBCC658	A87	nd	–
9	WRF	*Bjerkandera* sp.	FBCC395	BOS55	–	
10	WRF	*Botryobasidium vagum*	FBCC779	T130	–	
11	LDF	*Calocybe indica*	FBCC467	X28	–	
12	WRF	*Ceriporia viridans*	FBCC162	363	–	
13	WRF	*Physisporinus rivulosus*	FBCC939	T241i	–	–
14	WRF	*Cerrena unicolor*	FBCC744	T71	–	
15	WRF	*Chondrostereum purpureum*	FBCC814	T161-1i3	–	
16	LDF	*Clitocybe candicans*	FBCC628	K212	nd	+
17	LDF	*Clitocybe clavipes*	FBCC531	K71	+	
18	LDF	*Clitocybe lignatilis*	FBCC537	82	+	
19	LDF	*Clitocybula dusenii*	FBCC463	b12	–	
20	LDF	*Collybia confluens*	FBCC637	K246	–	
21	**LDF**	***Collybia peronata ****	FBCC635	K220	+++	++
22	LDF	*Collybia sp.*	FBCC613	K190	–	
23	WRF	*Conferticium ochraceum*	FBCC880	T194ia	+	
24	LDF	*Coprinus comatus*	FBCC568	K131	+	
25	[b]	*Crinipellis schevezenkovi*	FBCC657	A85	+	
26	WRF	*Cystostereum murrayi*	FBCC786	T140-lil	–	
27	WRF	*Dichomitus squalens*	FBCC184	PO114B	–	
28	LDF	*Flammulina velutipes*	FBCC583	K158	–	
29	LDF	*Galerina marginata*	FBCC1186	K96B	–	
30	LDF	*Galerina marginata*	FBCC1185	K96A	–	
31	WRF	*Ganoderma lipsiense*	FBCC133	334	+	
32	WRF	*Gelatoporia subvermispora*	FBCC314	CZ-3-FPL	–	
33	WRF	*Gelatoporia pannocincta*	FBCC185	PO115B	–	
34	BRF	*Gloeophyllum sepiarium*	FBCC75	125	+	
35	BRF	*Gloeophyllum trabeum*	FBCC328	R/83	–	
36	**LDF**	***Gymnopilus luteofolius ****	FBCC466	X9	++++	+++
37	**LDF**	***Gymnopilus penetrans ****	FBCC1010	HAM1	++	++++
38	**LDF**	***Gymnopus erythropus ****	FBCC1036	CCBAS 287 / PL49	+++	+
39	LDF	*Gyromitra esculenta*	FBCC639	K293	–	
40	WRF	*Haploporus odorus*	FBCC804	T154	–	
41	LDF	*Hygrophoropsis aurantiaca*	FBCC566	K123	+	
42	WRF	*Hyphoderma setigerum*	FBCC894	T204i	–	

	Group	Fungus	Strain	Former code	Growth in sawmill soil[a]	
					A	B
43	WRF-LDF	*Hypholoma capnoides*	FBCC541	K87B	–	
44	**WRF-LDF**	***Hypholoma fasciculare* ***	FBCC1034	CCBAS 281 / PL13	++++	++
45	WRF-LDF	*Hypholoma marginatum*	FBCC544	K92	–	
46	WRF	*Irpex lacteus*	FBCC1012	CCBAS 617/93	–	–
47	WRF	*Irpex lacteus*	FBCC384	CCB-196	–	–
48	WRF	*Junghuhnia collabens*	FBCC800	T152ia	–	
49	**WRF-LDF**	***Kuehneromyces mutabilis* ***	FBCC508	K22	++	–
50	LDF	*Lepiota cristata*	FBCC551	K104	–	
51	LDF	*Lepista nebularis*	FBCC550	K103	+	
52	LDF	*Leucopaxillus rhodoleuceus*	FBCC624	K207	–	
53	WRF	*Merulius tremellosus*	FBCC227	PO171i	–	
54	WRF	*Merulius tremellosus*	FBCC63	109	–	
55	WRF	*Merulius tremellosus*	FBCC362	77-51	–	
56	WRF	*Merulius tremellosus*	FBCC858	T186-1i1	–	
57	LDF	*Mycena amicta*	FBCC543	K91	+	
58	LDF	*Mycena epipterygia*	FBCC532	K72	–	
59	**LDF**	***Mycena galericulata* ***	FBCC598	K175	+++	+
60	LDF	*Mycena haematopus*	FBCC638	K292	–	
61	LDF	*Mycena polygramma*	FBCC615	K191	+	
62	LDF	*Mycena sp*	FBCC630	K215	–	
63	BRF	*Neolentinus lepideus*	FBCC321	V4/VTT	–	
64	BRF	*Neolentinus lepideus*	FBCC233	PO183	–	
65	WRF	*Panellus serotinus*	FBCC569	K133	+	
66	LDF	*Paxillus involutus*	FBCC234	PO184	–	
67	LDF	*Phaeolepiota aurea*	FBCC555	K111	–	
68	WRF	*Phanerochaete chrysosporium*	FBCC280	ME-446	–	
69	**WRF**	***Phanerochaete sanguinea* ***	FBCC319	221	–	–
70	WRF	*Phanerochaete sordida*	FBCC310	HHB-8922-sp	+	
71	WRF	*Phanerochaete sordida*	FBCC972	T272	–	
72	**WRF**	***Phanerochaete velutina* ***	FBCC941	T244i	+++	++++
73	WRF	*Phellinus laevigatus*	FBCC709	T45	–	
74	WRF	*Phlebia hydnoides*	FBCC364	74348-1	–	
75	WRF	*Phlebiella vaga*	FBCC878	T193i	–	
76	WRF	*Pholiota alnicola*	FBCC581	K153	–	
77	WRF	*Pholiota alnicola*	FBCC606	K185	–	
78	LDF	*Pholiota alnicola* var. a*lnicola*	FBCC542	K89	++	
79	LDF	*Pholiota lenta*	FBCC1013	CCBAS 456	–	
80	WRF-LDF	*Pholiota sp.*	FBCC529	K61	–	
81	WRF-LDF	*Pholiota sp.*	FBCC557	K113	++	+
82	WRF-LDF	*Pholiota sp.*	FBCC631	K216	++	+++
83	WRF	*Pholiota squarrosa*	FBCC552	K105	–	
84	**WRF**	***Physisporinus vitreus* ***	FBCC450	Hamburg	++	–
85	BRF	*Piptoporus betulinus*	FBCC13	29	–	

	Group	Fungus	Strain	Former code	Growth in sawmill soil[a]	
					A	B
86	BRF	*Piptoporus betulinus*	FBCC14	30	–	
87	BRF	*Piptoporus betulinus*	FBCC703	T35	–	+
88	WRF	*Pleurotus calyptratus*	FBCC675	A183	++	
89	WRF	*Pleurotus dryinus*	FBCC238	PO188	–	
90	WRF	*Pleurotus ostreatus*	FBCC375	DSM-11191	–	
91	WRF	*Pleurotus pulmonarius*	FBCC517	K42	+	
92	WRF	*Polyporus brumalis*	FBCC65	111	–	
93	[b]	*Polyporus sp.*	FBCC54	93	+	
94	**BRF**	***Postia caesia ****	FBCC757	T91	+	–
95	BRF	*Postia placenta*	FBCC287	280/VTT	–	
96	WRF	*Pycnoporus cinnabarinus*	FBCC130	331	–	
97	WRF	*Radulodon erikssonii*	FBCC752	T84	–	
98	WRF	*Resinicium furfuraeceum*	FBCC745	T74	–	
99	LDF	*Rhizoctonia praticola*	FBCC329	R/93	+	
100	**LDF**	***Rhodocollybia butyraceae ****	FBCC1035	CCBAS 286 / PL33	+++	++
101	LDF	*Rhodocollybia butyraceae*	FBCC626	K209	–	
102	BRF	*Serpula lacrymans*	FBCC1008	Md1	–	–
103	**BRF**	***Serpula lacrymans ****	FBCC1009	Md1144	–	–
104	WRF	*Sistotrema brinkmannii*	FBCC142	343	+	
105	WRF	*Skeletocutis kuehneri*	FBCC693	T23	+	
106	**WRF-LDF**	***Sphaerolobus stellatus ****	FBCC253	PO203	++++	++++
107	WRF	*Stereum hirsutum*	FBCC136	337	–	
108	**LDF**	***Stropharia aeruginosa ****	FBCC521	K47	++++	+
109	LDF	*Stropharia aeruginosa*	FBCC625	K208	–	++++
110	LDF	*Stropharia aeruginosa*	FBCC633	K218	++	–
111	LDF	*Stropharia ambigua*	FBCC480	TM 47-1 /stoc 1 gramB	+	+
112	LDF	*Stropharia hornemannii*	FBCC565	K122	++	–
113	LDF	*Stropharia hornemannii*	FBCC572	K138	++	+
114	LDF	*Stropharia hornemannii*	FBCC622	K205	–	++
115	LDF	*Stropharia rugosoannulata*	FBCC474	11373 G	+++	++
116	**LDF**	***Stropharia rugosoannulata ****	FBCC475	11372 B	++++	++
117	LDF	*Stropharia rugosoannulata*	FBCC486	Ho1	–	++
118	LDF	*Stropharia rugosoannulata*	FBCC490	Ho1	++	
119	LDF	*Stropharia semiglobata*	FBCC528	K60	–	
120	LDF	*Stropharia semiglobata*	FBCC534	K79	+	
121	WRF-LDF	*Thanateporus cucumeris*	FBCC485	C8	–	
122	WRF-LDF	*Thelephora terrestris*		PO187	–	
123	WRF	*Trametes gibbosa*	FBCC431	CBS-441.61	–	
124	WRF	*Trametes ochracea*	FBCC1011	HAM9	–	
125	WRF	*Trametes pubescens*	FBCC735	T65iB	–	
126	WRF	*Trametes velutina*	FBCC592	K169	–	
127	WRF	*Trametes versicolor*	FBCC275	205	+	
128	WRF	*Trametes versicolor*	FBCC496	221 SA	–	

	Group	Fungus	Strain	Former code	Growth in sawmill soil[a]	
					A	B
129	WRF	*Trichaptum pargamenum*	FBCC122	323	++	+
130	LDF	*Tricholoma album*	FBCC558	K115	–	
131	LDF	*Tricholomopsis rutilans*	FBCC511	K25	++	
132	[b]	unidentified	FBCC455	i63-2	–	
133	[b]	unidentified	FBCC235	PO185	–	
134	[b]	unidentified	FBCC236	PO186	–	
135	[b]	unidentified	FBCC153	354, PO85i1	–	
136	[b]	unidentified	FBCC244	PO194	–	
137	[b]	unidentified	FBCC246	PO196	–	
138	[b]	unidentified	FBCC247	PO197	–	
139	[b]	unidentified	FBCC248	PO198	+	
140	[b]	unidentified	FBCC249	PO199	+	
141	[b]	unidentified	FBCC250	PO200	+	
142	[b]	unidentified	FBCC251	PO201	+	–
143	[b]	unidentified	FBCC252	PO202	–	
144	[b]	unidentified	FBCC254	PO204	+	
145	[b]	unidentified	FBCC255	PO205	–	
146	[b]	unidentified	FBCC256	PO206	+	

* Selected fungus for agar plate screening test and enzymatic activity in pine bark.
[a] Fungal growth in sawmill soil A (9% organic matter) and B (84% organic matter): – negligible; + slight; ++ moderate; +++ intermediate; ++++ excellent
[b] not known.
WRF = white-rot fungus; BRF = brown-rot fungus; LDF = litter-decomposing fungus; WRF-LDF = fungus whose habitat overlaps with those of WRF and LDF.

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